The Regulation of Mast Cell Growth and Protease Expression by Cytokines.

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ABSTRACT OF THESIS

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In this study the diversity of mast cell proteases and some of the factors regulating mast cell growth and protease expression were examined.

Five proteases were isolated from mouse small intestinal mucosa and characterised in terms of their substrate specificities, substrate and inhibitor kinetics and immunohistochemical localisation. These studies revealed that the isolated proteases were all of mast cell origin and that they were chymotrypsin-like in their substrate specificities. The proteases were all identified as variants of mouse mast cell protease-1 which differed only in their carbohydrate moieties. Despite the fact that these enzymes shared a common core polypeptide they all differed significantly in the rate at which they hydrolysed low molecular weight synthetic substrates and in the rates at which they were inhibited by α_1-proteinase inhibitor. A similar, but distinct protease was isolated from peritoneal cavity mast cells of mice. This enzyme, also a chymase, had N-terminal sequence consistent with identification of the enzyme as mouse mast cell protease-4 which had previously only been identified by N-terminal sequence analysis of electro-blotted protein and from cDNA sequencing. This enzyme was not inhibited by, and actually degraded α_1-proteinase inhibitor.

Some of the factors which regulate mast cell growth, proliferation, survival and protease expression were examined in the rat. Stem cell factor (SCF) or cytokine-rich lymph node conditioned medium (LNCM) was administered to normal rats by intraperitoneal injection and the effects on a defined cell populations, the connective tissue mast cells (CTMC) of the peritoneal cavity were monitored. LNCM did not cause an increase in CTMC numbers but it did stimulate a switch in protease expression from rat mast cell protease I (RMCP I) alone to dual expression of both RMCP I and RMCP II. SCF alone caused a significant increase in CTMC numbers coupled with a decrease in RMCP I content and an increase in RMCP II content. Treatment with both LNCM and SCF together caused an even greater increase in both CTMC numbers and RMCP II expression than that seen with SCF alone. A second experiment was carried out where SCF was administered by daily intravenous injection for 14 days to both normal and parasitised rats and the effects of the treatment on CTMC and mucosal mast cell (MMC) populations was monitored. Intravenous SCF treatment resulted in a five-fold increase in peritoneal mast cell numbers with a concomitant decrease in RMCP I content. There was no significant expression of RMCP II in the CTMC of these animals.

An alternative approach in examining the role of SCF in regulating mast cell populations was the use of polyclonal antibodies raised against SCF to try and block SCF activity in both normal and parasitised rats.

In normal rats, treatment with anti-SCF antibodies caused an approximately 50% decrease in the number of mast cells detected in the peritoneal cavity and totally ablated the MMCs from the small intestinal mucosa. In parasitised rats treated with anti-SCF antibodies, the mucosal mast cell hyperplasia associated with infection was significantly delayed in rats infected with *Nippostrongylus brasiliensis* or *Trichinella spiralis* and completely ablated in rats infected with *Schistosoma mansoni*. The depletion of mast cells from the intestinal mucosa was accompanied by a reduction in the tissue concentration of RMCP II. When anti-SCF antibodies were administered to rats which had already developed a mast cell hyperplasia following *N. brasiliensis* infection, there was again a significant depletion of both mast cells and RMCP II from the small intestine.

A sensitive ELISA test was developed to measure soluble SCF in blood. This assay showed that circulating SCF levels were significantly increased by infection with both *N. brasiliensis* and *T. spiralis*. Taken together these results show that SCF and the cytokines in LNCM play an important role in the regulation of mast cell populations and their expression of proteases.

Declaration.

I hereby declare that:

1. This thesis has been composed by myself
2. The work described herein was carried out by myself or, where jointly, that fact has been acknowledged.
3. This thesis has not been the subject of any previous application.

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List of abbreviations

α_1-AC	α_1-Antichymotrypsin
α_1-PI	α_1 protease inhibitor
1,10 Phe	1,10 phenanthroline
2-ME	2-Mercaptoethanol
3,4-DCI	3,4-Dichloro-isocoumarin
ANOVA	Analysis of variance
BCA	Bicinechoninic acid
BMMC	Bone marrow-derived mast cell
BSA	Bovine serum albumin
BZ-DL-ARG-4-NPE	Benzoyl-DL-arginine-4-nitroanalide
BZ-L-TYR-4-NPE	Benzoyl-L-tyrosine-4-nitroanalide
CBZ-D-ALA-4-NPE	Carboxybenzoyl-D-alanine-4-nitrophenyl ester
CBZ-L-ALA-4-NPE	Carboxybenzoyl-L-alanine-4-nitrophenyl ester
CBZ-L-ARG-4-NPE	Carboxybenzoyl-L-arginine-4-nitroanalide
CBZ-L-PHE-4-NPE	Carboxybenzoyl-L-phenylalanine-4-nitrophenyl ester
CBZ-L-TRP-4-NPE	Carboxybenzoyl-L-tryptophan-4-nitrophenyl ester
CBZ-L-TYR-4-NPE	Carboxybenzoyl-L-tyrosine-4-nitrophenyl ester
cDNA	copy deoxyribonucleic acid
Con A	Concanavalin A
CTMC	Connective tissue mast cell
DFP	Diisopropylfluorophosphate
E^0	Initial active enzyme concentration
E64	N-[N-(L-3-Trans-carboxyoxirane-2 carbonyl)-L-leucyl] -agmatine
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ES	Enzyme/substrate complex
Fc_eRI	High affinity immunoglobulin E receptor
FITC	Fluorescein isothiocyanate

FPLC	Fast protein liquid chromatography
GM-CSF	Granulocyte-macrophage-colony stimulating factor
Hb	Haemoglobin
HBSS	Hank's balanced salt solution
Hct.	Haematocrit
HRPO	Horse radish peroxidase
I^0	Initial inhibitor concentration
ICAM	Intercellular adhesion molecule
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL-	Interleukin
IMDM	Iscove's modified Dulbecco's medium
k_{ass}	Association rate constant
k_{cat}	Catalytic constant (turnover number)
kDa	Kilo Dalton
K_i	True inhibition constant
K_i^{app}	Apparent inhibition constant
KiSV-MC	Kirsten sarcoma virus-immortalised mast cell
K_m	Michaelis constant
L_3	Third stage larvae
LNCM	Lymph node conditioned medium
MES	Morpholinoethanesulphonic acid
MLN	Mesenteric lymph node
MMC	Mucosal mast cell
MMCP-	Mouse mast cell protease
Mr	Molecular mass
mRNA	Messenger ribonucleic acid
MSMCP	Mouse serosal mast cell protease
MUTMAC	4-methyl umbelliferyl-p-(NNN trimethylammonium) cinnamate

Nb	*Nippostrongylus brasiliensis*
nkat	nano katal
OPD	Orthophenylenediamine
PBS	Phosphate buffered saline
PMC	Peritoneal mast cell
PMSF	Phenylmethylsulphonyl fluoride
PNGaseF	Peptide N glycosidase F
PVDF	Polyvinylidene difluoride
RBC	Red blood cell
RBL-	Rat basophilic leukaemia
RMCP I	Rat mast cell protease I
RMCP II	Rat mast cell protease II
rrSCF[164]	Recombinant rat stem cell factor [164]
s.e.m.	Standard error of mean
S^0	Initial substrate concentration
SCF	Stem cell factor
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Serpin	Serine protease inhibitor
SMCP-	Sheep mast cell protease
Succ-ala-ala-pro-phe-4NA	Succinyl-alanyl-alanyl-prolyl-phenylalanine-4-nitroanilide
SUCC-PHE-4-NPE	Carboxybenzoyl-L-phenylalanine-4-nitroanalide
$t_{1/2}$	Time to half inhibition
T20	Tween 20 (Polyoxyethylenesorbitan monolaurate)
t-PA	Tissue plasminogen activator
TRITC	Tetramethylrhodamine isothiocyanate
vcu	Villus/crypt unit
VIP	Vasoactive intestinal peptide
V_{max}	Maximum velocity
$^{w}/_{v}$	Weight/volume

WBC	White blood cell

Contents

Equations, Figures and Tables.

1. GENERAL INTRODUCTION

1.1 Mast Cells.

Mast cells are mononuclear granulocytes derived from bone marrow haemopoietic progenitor cells that express the high affinity $Fc_{\varepsilon}RI$ receptors for immunoglobulin E (IgE) on their surface, through which they may be activated (Ishizaka, Tomioka & Ishizaka, 1970). They are characterised by their content of numerous compact, metachromatic granules which have a high affinity for basic, thiazine and copper phthalocyanin dyes and have the capacity to synthesise, store and secrete a large array of highly potent mediators of both inflammatory and immune processes. A number of these mediators are pre-formed and are stored in the secretory granules. These include proteoglycans, biogenic amines, cytokines, glycosidases and proteases. In addition to the pre-formed mediators a number of mediators are synthesised *de novo* when the mast cells are activated, these include prostaglandins and leukotrienes which are metabolites of arachidonic acid, platelet activating factor and a range of cytokines.

Mast cells are found in all species of mammals, birds, reptiles, amphibians and fish so for investigated (reviewed by Riley, 1959; Selye, 1965; Chiu & Lagunoff, 1972), and are distributed throughout most tissues of the body, often in association with loose connective tissue elements or at mucosal surfaces, particularly in the gastrointestinal and respiratory tracts. Mast cell numbers are frequently increased in parasitic infections and allergic conditions and, together with IgE, are the fundamental components of immediate hypersensitivity reactions.

Ehrlich (1878) first used the term mast cell when he described cells with an "engorged" (mast) appearance, which contained numerous cytoplasmic granules with unique metachromatic dye-binding properties. Since that time the metachromatic staining properties of the granules has been considered the hallmark of the mast cell.

The mast cells located in connective tissue or mucosal sites, while sharing morphological features such as metachromatic cytoplasmic granules, differ in a number of aspects and are now considered to represent two distinct sub-sets which have been named connective tissue or mucosal mast cells depending on their anatomical site. There was considerable debate as to the existence of intestinal mucosal mast cells after they were first noted by Maximow (1906) who described cells which were smaller, less well granulated and poorly preserved compared with mast cells from other anatomical sites. Michels (1938) later described such cells as 'atypical' mast cells. Enerbäck (1966a) established that mucosal mast cells (MMC) in the intestinal mucosa of the rat had different morphology, sensitivities to fixation, dye binding properties and responsiveness to secretagogues compared with mast cells from other locations. The monoamine stored in MMC was also distinct from those present in 'typical' connective tissue mast cells (CTMC; Enerbäck, 1966a-d), and thus they represented two distinct sub-sets of mast cells.

One of the most reliable means of differentiating between MMC and CTMC is by their content of neutral serine proteases. In the rat CTMC contain a strongly basic, insoluble, chymotrypsin-like protease, rat mast cell protease (RMCP) I (Woodbury, Gruzensky & Lagunoff, 1978) whereas MMC contain the similar, but more soluble and antigenically distinct RMCP II (Woodbury, *et al*, 1978; Gibson & Miller 1986).

1.2 Proteoglycans

The characteristic metachromatic staining of mast cell granules with basic thiazine and copper phthalocyanin dyes, at low pH (≤ pH 0.5) indicates the presence of highly sulphated proteoglycans. In rodents, connective tissue-type 'typical' mast cells contain heparin proteoglycan (Enerbäck 1966b) whilst the 'atypical' mast cells located at or near mucosal surfaces contain predominantly chondroitin sulphate E (Enerbäck 1981). Both heparin and chondroitin sulphate E share a common core protein which contains a Ser-Gly-repeat sequence (Kjellen, Pettersson, Lillhager, Steen, Pettersson, Lehtonen, Karlsson, Ruoslahti & Hellman, 1989; Angerth, Huang, Aveskogh, Pettersson, Kjellen & Hellman, 1990) and differ in the composition of the repeating disaccharide units of their carbohydrate side chains which are attached to the serine residues of the core protein (Stevens, Lee, Seldin, Austen, Befus & Bienenstock, 1986). The presence of highly sulphated proteoglycan such as heparin in the CTMC of skin can be readily detected by staining with thiazine dyes such as toluidine blue or copper phthalocyanin dyes like astra blue at low pH (Enerbäck 1966b). When these dyes are used at pH 0.5 or less, only the strongly acidic sulphate groups remain ionised and thus capable of binding the dye (Enerbäck 1966b). However it is clear that the MMC in the small intestine of the rat differ from those in skin since their staining properties with the above techniques is dependent on the type of fixative used in the preparation of the tissue sections (Enerbäck 1966b).

1.3 Mast cell hyperplasia in parasitic infection.

Mast cell hyperplasia in association with parasitic helminth infection was first observed in the gastrointestinal tract of rats by Taliaferro & Sarles (1939) who found

increased numbers of globule leukocytes and mast cells in the small intestine of rats infected with *Nippostrongylus muris* (later re-named *N. brasiliensis)*. Since that time mast cell proliferation in the gastrointestinal mucosa has been recognised as a characteristic feature of enteric parasitism in many species, including man (reviewed in Miller, 1992). In murine rodents these mast cells have morphological, histochemical and fixation properties which identify them as mucosal mast cells (Miller & Walshaw 1972; Enerbäck, 1981).

1.4 The activation of mast cells and their possible role in the rejection of parasites.

Despite the fact that the mast cell hyperplasia in response to parasitic infections has been recognised since the late 1930s the role they play in the hosts immune response is not well understood and indeed there has been considerable debate as to whether they have a significant role at all. Many studies have shown that parasite-specific IgE is produced in response to infection (Jarrett & Miller 1982; Rothwell 1989) and that immediate hypersensitivity reactions are involved in the protective response (Miller, Woodbury, Huntley & Newlands, 1983). In immediate hypersensitivity-type reactions allergen-specific IgE binds to the high affinity IgE receptor ($Fc_{\varepsilon}RI$) which is expressed on the surface of mast cells and basophils. When IgE molecules, bound in this way, are cross-linked by interaction with specific allergen or with anti-IgE antibody, several processes are initiated. The mast cells are stimulated to secrete pre-formed mediators such as histamine and proteases (Wyczolkowska, Brzezinskablaszczyk & Maslinski, 1981, Miller *et al* 1983). Cross-linking of surface-bound IgE also initiates signal transduction pathways which can

result in *de novo* synthesis of numerous cytokines (Gordon, Burd & Galli, 1990) and arachidonic acid metabolites e.g. prostaglandin D_2 (Lewis, Soter, Diamond, Austen, Oates & Roberts, 1982) through the cyclo-oxygenase pathway, and leukotriene C_4, (Razin, Menciahuerta, Lewis, Corey & Austen, 1982) through the lipoxygenase pathway.

Evidence for activation of MMC *in vivo* is provided by the anaphylactic release of RMCP II into both the blood and gut lumen of the rat following intravenous treatment of primed (immune) rats with soluble worm antigen (King & Miller, 1984; King, Miller, Woodbury & Newlands, 1986) which strongly suggests that the mast cells are being activated through surface-bound IgE. It is also clear that mast cells are functionally active during the period when parasites are expelled by their host. This is shown most clearly by the systemic secretion of mast cell-specific proteases into the blood of parasitised rats and mice during worm expulsion (Woodbury, Miller, Huntley, Newlands, Palliser & Wakelin, 1984).

However, it may be that mast cells are not always an essential component of the immune response to helminths. For example, there are substantial accumulations of mast cells around the sites of persistent cestode infections in rats (Lindsay & Williams 1985; Chernin, Miller, Newlands & McLaren, 1988) which have no apparent detrimental effect on the parasites. In addition, both mast cell deficient W/W^v mice and W^s/W^s rats expel the intestinal nematode *N. brasiliensis* at virtually the same rate as their congenic +/+ littermates in the absence of any significant mastocytosis (Reed 1989; Arizono, Kasugai, Yamada, Okada, Morimoto, Tei, Newlands, Miller & Kitamura, 1993). The involvement of mast cells in the mouse may also be, at least to

some extent, dependent on the species of parasite since, in contrast with *N. brasiliensis*, expulsion of *Trichinella spiralis* is significantly delayed in W/W^v mice (Reed 1989) and totally abrogated in normal mice treated with anti-*c-kit* antibodies which abolished the mast cell hyperplasia associated with infection (Grencis, Else, Huntley & Nishikawa, 1993).

1.5 Regulation of mast cell differentiation and hyperplasia

There are at least two distinct mechanisms which regulate mast cell proliferation, differentiation and survival in rats and mice. One of these is mediated by stem cell factor (SCF) a cognate ligand for *c-kit*, which is a tyrosine kinase receptor that is encoded by the *c-kit* proto-oncogene (Williams, Fletcher, Lyman & de-Vries, 1991; Besmer 1991; Galli, Zsebo & Geissler, 1994; Kitamura, Tsujimura, Jippo, Kasugai & Kanakura, 1995). SCF is a growth factor produced by many cells including fibroblasts and other stromal cells, some epithelial cells and vascular endothelial cells (Galli *et al* 1994; Kitamura *et al* 1995). This growth factor has multiple activities that influence the development of $c\text{-}kit^+$ lineages, including haemopoietic progenitor cells and mast cells as well as primordial germ cells, melanocytes and certain other cell types (Williams *et al* 1991; Besmer 1991; Galli *et al* 1994). SCF is also known as Steel factor, kit-ligand and mast cell growth factor (Williams *et al* 1991; Besmer 1991; Galli *et al* 1994). SCF is the designation that will be used in this thesis. Several lines of evidence indicate that, under physiological conditions *in vivo*, SCF is critical for the development and survival of all mouse and rat mast cells, including CTMCs and MMCs (Galli *et al* 1994; Kitamura *et al* 1995). The other mechanism that regulates mast cell development in mice and rats is mediated by T cells and possibly

other sources of interleukin-3 (IL-3), IL-4, IL-9 and IL-10 which can promote and/or co-promote the proliferation and/or survival of certain murine mast cell populations including the MMC-like bone marrow-derived mast cells (BMMC) that can be generated *in vitro* (Galli *et al* 1994; Kitamura *et al* 1995; Schmitt, Huls, Nagel & Rude, 1990; Thompson-Snipes, Dhar, Bond, Mosmann, Moore & Rennick, 1991).

In murine rodents, infection with intestinal nematodes induces a striking hyperplasia of intestinal MMCs, which is accompanied by increases in the levels of MMC-associated proteases in intestinal tissues and increases in the concentrations of these proteases in the blood (Miller & Jarrett 1971; Miller *et al* 1983; Huntley, MacKellar, Newlands, Irvine & Miller, 1990; Huntley, MacKellar & Miller, 1993; Woodbury *et al* 1984). Previous work indicated that both SCF and T cell-dependent mechanisms contribute to the intestinal MMC hyperplasia associated with nematode infection. In comparison to normal mice or rats, those with mutations that markedly diminish *c-kit* receptor tyrosine kinase activity, such as W/W^v mice (Reed 1989) or W^s/W^s rats (Arizono *et al* 1993), exhibit no or greatly diminished hyperplasia of intestinal MMCs in response to infection with *N. brasiliensis* (Reed 1989; Arizono *et al* 1993), or *T. spiralis* (Reed 1989). Both intestinal MMC hyperplasia and the spontaneous expulsion of the parasites are diminished in *T. spiralis*-infected mice that have been treated with an antibody to the *c-kit* receptor (Grencis *et al* 1993). Moreover, treatment of normal rats with *E. coli*-derived recombinant rat SCF^{164}, which represents virtually the entire extracellular ligand domain of SCF and which possesses high biological activity (Galli *et al* 1994), induces significant hyperplasia of gastrointestinal MMCs as well as connective tissue-type mast cells (Tsai, Shih, Newlands, Takeishi, Langley, Zsebo, Miller, Geissler & Galli, 1991). The T cell-dependent nature of the other mechanism, on the other hand, is demonstrated in athymic

'nude' mice which fail to develop MMC hyperplasia in response to nematode infection. This response is restored in nude animals treated by adoptive transfer of T cells from congenic normal +/+ littermates (Reed 1989). Moreover, in normal mice, treatment with either anti-IL-3 or anti-IL-4 antibodies, or, even more effectively, treatment with both antibodies together, significantly suppresses intestinal mast cell hyperplasia in response to *N. brasiliensis* infection (Madden, Urban, Ziltener, Schrader, Finkelman & Katona1, 1991). Finally, *in vitro* evidence indicates that SCF and IL-3 can have synergistic effects in promoting the proliferation of rat mast cells with phenotypic similarities to MMCs (Haig, Huntley, MacKellar, Newlands, Inglis, Sangha, Cohen, Hapel, Galli & Miller, 1994; Tei, Kasugai, Tsujimura, Adachi, Furitsu, Tohya, Kimura, Zsebo, Newlands, Miller, Kanakura & Kitamura, 1994).

Taken together, this evidence indicates that the cytokine-dependent regulation of MMC hyperplasia during nematode infection in murine rodents, while complex, importantly involves both SCF and T cell-derived cytokines such as IL-3 and IL-4.

1.6 Mast cell proteases

The first indication that mast cells contained proteases was the histochemical localisation of esterase activity in the mast cells of skin by Gomori (1951). This was confirmed some years later by the work of Benditt & Arase (1959) who found enzymic activity similar to α-chymotrypsin in isolated rat peritoneal mast cells. Mast cell granules, in fact, store a number of different types of proteases (Table 1). A major granule component in rodents and man is the zinc metalloexopeptidase, carboxypeptidase A (Reynolds, Stevens, Gurley, Lane, Austen & Serafin, 1989; Goldstein, Kaempfer, Proud, Schwartz, Irani & Wintroub, 1987).

Table 1 Mast cell serine proteases.

Species	Protease	Specificity	Location
Mouse	MMCP-1	chymase	MMC
	MMCP-2	chymase	MMC
	MMCP-3	chymase	CTMC ?
	MMCP-4	chymase	CTMC
	MMCP-5	chymase	CTMC
	MMCP-6	tryptase	CTMC
	MMCP-7	tryptase	CTMC
Rat	RMCP I	chymase	CTMC
	RMCP II	chymase	MMC
	RMCP 3	chymase	RBL-1
	RMCP 4	chymase	RBL-1
	RMCP 5	chymase	CTMC
	RMCP 6	tryptase	CTMC
	RMCP 7	tryptase	CTMC
	RMCP 8	?	RBL-1(?MMC)
	RMCP 9	?	RBL-1(?MMC)
	RMCP 10	?	RBL-1(?MMC)
Human	Chymase	chymase	CTMC
	Tryptase	tryptase	MMC/CTMC
Dog	tryptase A	tryptase	?
	tryptase B	tryptase	?
	chymase	chymase	?
Sheep	SMCP 1	chymase/tryptase	MMC
	SMCP 2	chymase ?	CTMC ?

* Much of the work on canine mast cell proteases has been carried out on mastocytoma-derived material and there is no definitive information available on the distribution of the proteases. RBL-1 is a rat basophilic leukaemia cell line with many similarities to MMC.

Neutral serine proteases have also been identified as major secretory granule components in the mast cells of several species including mouse (Dubuske, Austen, Czop & Stevens, 1984; Newlands, Gibson, Knox, Grencis, Wakelin & Miller, 1987), rat (Lagunoff & Pritzl 1976; Woodbury, Gruzenski & Lagunoff, 1978), dog

(Caughey, Viro, Ramachandran, Lazarus, Borson & Nadel, 1987; Vanderslice, Craik, Nadel & Caughey, 1989) sheep (Huntley, Gibson, Knox & Miller, 1986; McAleese, Pemberton, Huntley and Miller, unpublished) and man (Schwartz, Lewis & Austen, 1981; Schechter, Choi, Slavin, Deresienski, Sayama, Dong, Lavker, Proud & Lazarus, 1986). The distinctive, apparently tissue-specific, distribution of mast cell proteases in man and rodents suggests that different mast cell populations may respond according to the type of protease secreted.

The chymotrypsin-like proteases found in mast cell granules are structurally similar to the granule-associated proteases, such as cathepsin G, granzyme B and granzyme H, found in other cell types of haemopoietic origin like T cells and neutrophils (Reviewed by Caughey 1995). These proteases are of similar size, 30 - 35 kDa and each contain 6 half-cystein residues indicating the probable presence of 3 intra-chain disulphide bonds (Le Trong, Parmelee, Walsh, Neurath & Woodbury, 1987). Bovine pancreatic chymotrypsin and a number of plasma serine proteases differ from the secretory granule-associated proteases in that they have an additional disulphide bond in the active site-substrate binding region (Le Trong *et al* 1987). In both mouse and man the genes which encode these granule-associated proteases are clustered on chromosome 14 (Gurish, Nadeau, Johnson, McNeil, Grattan, Austen & Stevens, 1993; Hohn, Popescu, Hanson, Salvesen & Ley, 1989) suggesting that they may have arisen by gene duplication fairly recently in evolutionary terms. This is supported by the similarities in the basic organisation of the introns and exons of the genes which encode these proteases in that they share a common I, II, 0, 0 intron phase pattern (reviewed by Caughey 1995).

Rat CTMC, typified by those found at serosal surfaces, contain the chymotrypsin-like enzyme rat mast cell protease I (RMCP I; Lagunoff & Pritzl 1976) while MMC, found predominantly in the mucosa of the gastrointestinal tract, contain the similar, but antigenically distinct and more soluble enzyme, rat mast cell protease II (RMCP II; Woodbury, *et al* 1978). These proteases share 74% amino acid sequence identity (Le Trong *et al* 1987) but have substantially different physical and enzymic properties. RMCP I is insoluble in physiological conditions, requiring 1 M NaCl to solubilise it (Lagunoff & Pritzl 1976) whereas RMCP II is readily soluble (Woodbury *et al* 1978). These properties are due, in part at least, to the net charge of the proteases. RMCP I is highly basic with a net charge of +18 whilst RMCP II has a net charge of +3 (Le Trong *et al* 1987). In terms of function both proteases have chymotrypsin-like substrate specificities (Lagunoff & Pritzl 1976; Woodbury *et al* 1978) but RMCP II is efficiently inhibited by the plasma serine protease inhibitor α_1-protease inhibitor (α_1-PI) while RMCP I is not inhibited by, and in fact hydrolyses, α_1-PI (Pirie-Shepherd, Miller & Ryle, 1991). Quantitation of the distribution and of the systemic secretion of RMCP II during intestinal immunological reactions has been facilitated by the development of highly sensitive enzyme linked immunosorbent assays (ELISA; reviewed in Miller, Huntley, Newlands & Irvine, 1990) and it is clear from these studies that RMCP II serves a very different function from that of RMCP I, for example it is synthesised and secreted in large amounts during parasitic infections, whereas concentrations of RMCP I remain relatively unchanged (Miller *et al* 1990).

The genes encoding a number of other serine proteases have been cloned from isolated peritoneal mast cells or from rat basophilic leukaemia (RBL) cell lines. In addition to RMCP I and II, which are well characterised, RMCP 3-10 have now been identified (Lutzelschwab, Pejler, Aveskogh & Hellman, 1997). RMCP-3 and -4, which were cloned from RBL-1 cells and have been classified as chymases, do not appear to be transcribed in either normal rat peritoneal mast cells or intestine (Lutzelschwab *et al* 1997). RMCP-5, also a chymase originally described by Ide and colleagues (Ide, Itoh, Tomita, Murakumo, Kobayashi, Maruyama, Osada & Nawa, 1995) as RMCP-3 is predominantly expressed in CTMC but is also expressed, at low levels, in normal intestine (Lutzelschwab *et al* 1997). RMCP-6 and -7 were classified as tryptases, i.e. they have trypsin-like substrate specificities, and appear to be expressed exclusively in CTMC (Lutzelschwab *et al* 1997). RMCP-6 is the rat mast cell tryptase identified by Lagunoff, Rickard & Marquardt (1991). RMCP-8, -9 and -10 appear to form a sub-family which may be more closely related to the T cell granzymes than to the other mast cell proteases (Lutzelschwab *et al* 1997) and their substrate specificities have not yet been determined. The RMCP-8 sub-family of enzymes were cloned from RBL-1 cells and are expressed in normal rat intestine (Lutzelschwab *et al* 1997).

Tryptases have also been found in the mast cells of several other species including mouse, dog and man (Reynolds, Stevens, Lane, Carr, Austen & Serafin, 1990; Caughey *et al* 1987; Schwartz *et al*, 1981).

Mouse mast cell protease (MMCP-1) isolated from the intestinal mucosa of mice infected with the parasite *T. spiralis*, shares 74% amino acid sequence homology with RMCP II and also has a very similar tissue distribution and biochemical

properties (Newlands *et al.*, 1987; Miller, Huntley, Newlands, MacKellar, Lammas & Wakelin, 1988; Le-Trong, Newlands, Miller, Charbonneau, Neurath & Woodbury, 1989; Huntley, Gooden, Newlands, MacKellar, Lammas, Wakelin, Tuohy, Woodbury & Miller, 1990). Murine bone marrow-derived mast cells (BMMC), often regarded as in vitro analogues of MMC (Sredni, Friedman, Bland & Metcalfe, 1983), contain four proteases in the molecular mass range 28-32 kDa including MMCP-1 as determined by Western blotting and ELISA (Newlands, Lammas, Huntley, MacKellar, Wakelin & Miller, 1991). Four esterases of similar or identical molecular weight which bind the specific serine protease inhibitor [^{3}H]-diisopropylfluorophosphate (DFP) have also been found in BMMC (DuBuske *et al.*, 1984). Five proteases have been identified in CTMC and/or virus-immortalised mast cell lines on the basis of N-terminal amino acid sequences and molecular weights and designated MMCP-2 to MMCP-6 (Serafin, Reynolds, Rogelj, Lane, Conder, Johnson, Austen & Stevens, 1990; Reynolds *et al*, 1990). All but one of these proteases, MMCP-3, have been cloned and sequenced. MMCP-1 is expressed only in MMC and MMCP-2 is expressed in intestinal mucosa and Kirsten sarcoma virus-immortalised mast cells (KiSV-MC; Serafin, Sullivan, Conder, Ebrahimi, Marcham, Johnson, Austen & Reynolds, 1991). MMCP-4 is transcribed in CTMC and KiSV-MC (Serafin *et al* 1991) and MMCP-5 in CTMC and KiSV-MC (Huang, Blom & Hellman, 1991; McNeil, Austen, Somerville, Gurish & Stevens, 1991). MMCP-6, identified as a tryptase by its predicted amino acid sequence, appears to be exclusive to CTMC and KiSV-MC (Reynolds, Gurley, Austen & Serafin, 1991). A second tryptase, MMCP-7 has been identified and found to be transiently expressed in immature, cultured

BMMC maintained in IL-3 (McNeil, Reynolds, Schiller, Ghildyal, Gurley, Austen & Stevens, 1992). This tryptase has amino acid sequence homologies in the range 71-76% with MMCP-6, dog and human tryptases but differs in its gene structure from MMCP-6, having only 5 exons rather than the 6 in MMCP-6 (McNeil *et al* 1992).

1.7 Mast cell proteases; regulation of synthesis.

Rat BMMC, grown in IL-3 synthesise and store abundant RMCP II in their granules (Haig, McMenamin, Redmond, Brown, Young, Cohen & Hapel, 1988) and it is reasonable to suggest that IL-3 is responsible for regulating RMCP II synthesis in the rat. However, co-culture of rat BMMC in both IL-3 and SCF depressed RMCP II expression compared with IL-3 alone and did not induce expression of RMCP I (Haig *et al* 1994). Thus it may be that IL-3 and SCF have opposite regulatory roles in the protease expression of rat MMC. In contrast, when rat CTMC are co-cultured in IL-3 and SCF they switch from expressing RMCP I alone to co-expressing both RMCP I and RMCP II (Haig *et al* 1994). On the other hand *in vitro* murine BMMC grown in IL-3 express little or no MMCP-1 (Ghildyal, McNeil, Gurish, Austen & Stevens, 1992), the mouse analogue of RMCP II, and are not induced to express this protease until they are exposed to IL-10 (Ghildyal *et al* 1992). The BMMC grown in IL-3-enriched medium express high steady-state levels of mRNA encoding for MMCP-5 but not MMCP-1, MMCP-2 or MMCP-4 (Xia, Ghildyal, Austen & Stevens, 1996). However, nuclear run-on analysis shows that the genes for all four chymases are transcribed (Xia *et al*, 1996). BMMC grown with the addition of IL-10 to the culture medium, express high steady-state levels of the transcript encoding for MMCP-2 and, in pulse-chase experiments, the half life of the MMCP-2 transcript was ~ 4-fold longer

than in replicate cells subsequently grown in the absence of IL-10 (Xia *et al*, 1996). Experiments with cycloheximide and actinomycin D indicate that IL-10 induces expression of a *trans*-acting factor(s) which stabilise the MMCP-2 transcript or in some other way facilitate its processing (Xia *et al* 1996). When murine BMMC were exposed to SCF they also expressed MMCP-4, the mouse analogue of RMCP I, showing clearly that there are major differences between the two species in the mechanism by which their protease expression is regulated.

1.8 Mast cell proteases; function.

Although mast cell proteases, particularly the chymases RMCP II in the rat and MMCP-1 in the mouse, are synthesised and secreted in large quantities during parasitic infections there is relatively little known about their *in vivo* function or their native substrates. There is, however, some indication of the possible *in vivo* role of the mast cell proteases in general from *in vitro* and *ex vivo* experiments. Human mast cell tryptase has mitogenic effects on cultured fibroblasts (Ruoss, Hartmann & Caughey, 1991), tracheal smooth muscle (Brown, Tyler, Jones, Ruoss, Hartmann & Caughey, 1995) and bronchial epithelial cells *in vitro* (Cairns & Walls, 1996). The mechanism by which tryptase stimulates growth is not certain but probably involves cleavage of protease-activated receptors on the cell surface. A receptor (PAR-2) has been identified in both mouse and humans which, when activated through cleavage by trypsin stimulates cell growth and proliferation (Nystedt, Emilsson, Wahlestedt & Sundelin, 1994: Nystedt, Emilsson, Larsson, Strombeck & Sundelin, 1995). Thus far, these 'tethered' receptors have been found to be expressed on endothelial cells, fibroblasts, keratinocytes and neutrophils (Storck, Kusters, Vahland, Morys-

Wortmann & Zimmermann, 1996; Hou, Macey, Fox, Stone & Howells, 1996; Howells, Macey, Hou & Stone, 1997). No such mitogenic function has been ascribed to mast cell chymase but it is interesting to note that the sheep mast cell protease, SMCP-1 has dual chymase and tryptase substrate specificities (Pemberton, Huntley & Miller 1997), a characteristic it shares with cathepsin G, another granulocyte-associated serine protease (Hof, Mayr, Huber, Korzus, Potempa, Travis, Powers & Bode, 1996) suggesting that it could fulfil such a role. Chymases such as RMCP II will degrade type IV collagen, a component of basement membrane (Sage, Woodbury & Bornstein, 1979; Katunuma & Kido, 1988) and are able to activate other proteases such as the matrix metallo-proteinases gelatinase (Gruber & Schwartz, 1990) and stromelysin (Gruber, Marchese, Suzuki, Schwartz, Okada, Nagase & Ramamurthy, 1989), through proteolytic processing of the pro-enzymes (Suzuki, Lees, Newlands, Nagase & Woolley, 1995) suggesting that it may have a role in remodelling and/or wound repair.

Proteases may also have an important role in regulating inflammatory processes. Human tryptase, for example, up-regulates synthesis and secretion, by epithelial cells (Cairns & Walls), of IL-8, a chemo-attractant for neutrophils (Parsons, Fowler, Heyers & Henson, 1985), and also increases epithelial expression of the intercellular adhesion molecule ICAM-1 (Cairns & Walls, 1996) which has an important role in recruitment of leukocytes to airways (Wegner, Gundel, Reilly, Hayes, Letts & Rothlein, 1987). On the other hand, tryptase and chymase are reported to degrade a number of regulatory neuropeptides such as vasoactive intestinal peptide (VIP) and substance P (Caughey, Leidig, Viro & Nadel, 1988) which have pro-inflammatory

activities in that they can stimulate release of the biogenic amines histamine and serotonin from mast cells (Irman-Florjanc & Erjavec, 1983) as well as direct effects on smooth muscle tone (Caughey *et al* 1988).

More recently *ex vivo* perfusion studies have shown that RMCP II is released in a steady-state manner into the vasculature by the intestinal mast cells of rats (Scudamore, Pennington, Thornton, McMillan, Newlands & Miller, 1995). The rate of protease release was significantly higher in rats primed by previous infection with *N. brasiliensis* than that detected in uninfected controls, reflecting the higher mast cells numbers present in the primed animals (Scudamore *et al* 1995a). The concentration of RMCP II in vascular perfusate increased by 1000 fold within 2 minutes after the introduction of soluble *N. brasiliensis* antigen into the vascular perfusate (Scudamore, Thornton, McMillan, Newlands & Miller, 1995b). After its release the RMCP II rapidly reaches the gut lumen (Scudamore *et al* 1995a). This process was accompanied by an increase in both vascular and epithelial permeability which allowed the translocation of Evan's blue-labelled human serum albumin from the vascular perfusate to the gut lumen (Scudamore, *et al* 1995b). The rapid development of this macromolecular permeability of the vascular endothelium and mucosal epithelium, in the absence of any gross epithelial lesions, suggests that RMCP II increases permeability via a paracellular route by attacking the epithelial junctional complex (Scudamore *et al* 1995b).

1.9 Aims of this thesis.

The mast cell proteases RMCP I and II, in the rat and MMCP-1, in the mouse, have for some time, served as important markers of mast cell phenotype. There is now,

however, a considerable body of data which shows that these proteases are only part of substantial multi-enzyme families. Much of the data on the diversity of rodent mast cell proteases has been obtained by cloning and sequencing the genes which encode for these proteases and relatively little is known about the functional properties of these enzymes or about the mechanisms which regulate their expression *in vivo.* MMCP-1 has been isolated from mouse intestine and partially characterised (Newlands *et al* 1987) and there is now substantial evidence for a number of other mast cell protease in the intestinal mucosa. The first aim of this study will be to investigate the diversity of intestinal mast cell proteases in the mouse by isolating and characterising the proteases present.

Mast cell proliferation, differentiation, survival and protease expression in rats and mice is regulated through stem cell factor, derived from connective tissue elements such as fibroblasts, and through the T cell-derived cytokines IL-3, -4, -9 and -10. In this laboratory we have developed highly specific probes for the identification and localisation of RMCP I and RMCP II which are expressed in CTMC and MMC respectively. This means that the rat makes a suitable model for studying the effects of cytokines on these mast cell sub-populations and on their protease expression. This will be addressed by examining the effects of exogenously administered SCF or cytokine-rich LNCM to normal animals and monitoring the effects on defined cell populations, the CTMC of the peritoneal cavity and MMC in intestinal mucosa. Another approach to examining the role of SCF in regulating mast cell populations will be the preparation of polyclonal antibodies to SCF and the use of these antibodies to try and block SCF activity *in vivo*. This will be carried out, initially, in normal rats.

Since mast cell hyperplasia is a prominent feature associated with intestinal nematodiasis this procedure will be extended to examine the effects of polyclonal anti-SCF on mast cell populations and mast cell protease expression in parasitised rats.

2. MATERIALS AND METHODS

2.1 Mouse Mast Cell Protease-1 Glycoforms.

2.1.1 Animals and parasitic infection.

Male and female NIH mice, 8 to 10 weeks old, bred and maintained under conventional conditions at the Department of Zoology, University of Nottingham, were infected with 300 *Trichinella spiralis* muscle larvae as described previously (Wakelin & Lloyd 1976). Female Balb/c mice, 6 weeks old, were infected with 300 *Nippostrongylus brasiliensis* L_3 by subcutaneous injection.

2.1.1.1 Culture of *N. brasiliensis.*

N. brasiliensis were cultured, by the following technique, by passage through Wistar rats, maintained under conventional conditions at the Moredun Research Institute, Edinburgh. Rats were infected with ~ 3000 third stage larvae (L_3) by subcutaneous injection and faeces from the rats was collected onto moist paper towels, placed on a tray under the cage, between 7 and 9 days after primary infection. The faecal pellets were soaked in tap water for approximately 1 hour after collection and then thoroughly mixed with gas absorption grade, granular activated charcoal (BDH, Poole, U.K.) in approximately equal volumes. The mixture was spread onto moist, 7 cm diameter filter paper leaving a 1 cm clear margin, placed in a Petri dish and incubated at 25°C for 5 to 7 days. Infective L_3 migrated to the edge of the filter paper where they were harvested by half filling the Petri dish with water at 37°C and

incubated at that temperature for 5 min, during which time the larvae migrated into the water. The larval suspension was decanted into a Baerman apparatus lined with K-Dex paper (Kleenaroll Ltd. London, U.K.) and filled with water at 37°C. Viable larvae actively migrated through the paper and settled out at the bottom of the Baerman funnel after about 1 hour. The larvae were collected and concentrated by centrifugation at 100 xg for 3 min and subsequently washed x3 with physiological saline containing penicillin (100 units/ml) and streptomycin (10 units/ml).

2.1.1.2 Calculation of larval dose.

Three 50 µl aliquots of the washed larval suspension were each spotted onto microscope slides in a series of small drops and the larvae counted using a dissecting microscope. The mean of the three counts was taken and the larval dose adjusted to the desired volume.

2.1.1.3 Material collected post mortem.

The mice were killed by cervical dislocation 10 days after infection. The small intestine of each mouse was removed and the lumen flushed through with Hanks balanced salt solution (HBSS) to remove digesta before storage at -20°C or fixation in Carnoy's fluid and subsequent processing to paraffin wax for immunohistochemistry as described previously (Newlands, MacKellar & Miller, 1990). Porton mice, 6 months old, bred and reared at Moredun Research Institute were used for isolation of murine serosal mast cell protease (MSMCP).

2.1.2 Isolation of the variant forms of mouse mast cell protease-1 from small intestine.

Murine intestinal mast cell proteases were isolated by a modification of the previously published method (Newlands *et al.*, 1987). Briefly, after thawing, 10 g of small intestine was chopped finely with scissors and homogenised in 20 mM Tris/HCl pH 7.5 (3 ml per gram) with a Polytron homogeniser (Northern Media Supplies Ltd. North Cave, North Humberside, UK.). The homogenate was centrifuged at 5000 xg for 5 min and the supernatant was centrifuged at 50,000 xg for 15 min. The final supernatant was applied to a 16 x 65 mm CM-Sepharose cation exchange column (Pharmacia, Milton Keynes, UK) equilibrated with 20 mM Tris/HCl pH 7.5. Bound proteins were eluted with a 0-1.0 M NaCl gradient over 90 ml. Fractions (5 ml) were collected and screened for chymase activity against the synthetic substrate CBZ-L-Tyrosine 4-nitrophenyl ester (CBZ-L-Tyr-4NPE) (Sigma) as described previously (Knox, Gibson & Huntley, 1986). Those fractions which contained enzyme activity were pooled and applied to a 16 x 160 mm column of Sephadex G25 (Pharmacia) to rapidly desalt and exchange the buffer for 50 mM morpholinoethanesulphonic acid (MES; Sigma) pH 6.0. The desalted material was applied to a Mono-S cation exchange column (Pharmacia) equilibrated with 50 mM MES pH 6.0 and eluted with a 50 mM-150 mM NaCl gradient over 20 ml. Individual peaks were collected and those with activity against CBZ-L-Tyr-4NPE were diluted 5 fold with 50 mM MES pH 6.0, re-applied to Mono-S and again eluted with 50-150 mM NaCl gradient.

2.1.3 Serosal Mast Cell Protease

Mice were killed by cervical dislocation following anaesthesia with Halothane (May and Baker) and cells recovered from the peritoneal cavity by lavage with 5 ml HBSS containing 0.1% W/v gelatin (Hanks/gelatin). Cells were sedimented by centrifugation (400 xg for 10 min), washed once with Hanks/gelatin and re-suspended in 1 ml Hanks/gelatin. Mast cell numbers were evaluated using a haemocytometer after staining with 1% W/v methylene blue in 50% V/v propylene glycol. The cell suspension was again sedimented by centrifugation, the supernatant fluid discarded, and the cell pellet stored at -20°C. A mast cell granule preparation was made after the method of Lagunoff & Pritzl (1976). Briefly, a thawed cell pellet was re-suspended in 1 ml distilled water and the cells fully disrupted by sonication (MSE, England) at 16 μm amplitude for 3 x 10 sec. with a 3 mm diameter probe. Granules were isolated from the lysate by a differential centrifugation technique, first at 200 xg to remove nuclei and larger cell debris followed by centrifugation at 50,000 xg for 15 min to sediment the mast cell granules. The granule pellet was washed x2 with PBS and finally solubilised in 1 ml of 2 M NaCl. The pellet extract was further centrifuged at 8000 xg for 2 min on an Eppendorf bench-top centrifuge and the supernatant fluid applied to a Sephacryl-S200 (Pharmacia) size exclusion column (10 x 200 mm) equilibrated with 1.0 M NaCl in 20 mM tris/HCl pH 7.5. Fractions (1 ml) were collected and tested for enzyme activity against CBZ-L-Tyr-4NPE. Those containing activity were pooled, diluted to a final concentration of 0.5 M NaCl with 20 mM tris/HCl pH 7.5 and applied to the Mono-S cation exchange column equilibrated with 0.5 M NaCl in 20 mM tris/HCl pH 7.5. The column was eluted with a 0.5 - 1.0 M

NaCl gradient over 10 ml. All centrifugation and extraction steps were carried out at +4°C.

2.1.4 Protein estimations

Protein concentrations were estimated using a Pierce BCA protein assay kit (Pierce and Warriner, Chester, UK), using bovine serum albumin as standard, in accordance with the manufacturer's instructions.

2.1.5 SDS-PAGE

Discontinuous SDS-PAGE was carried out as described by Laemmli (1970) on 15% mini slab gels (Mini Protein II, BioRad) run at 200 volts constant for 1.5 h or on large format (Protean II xi, BioRad) 10% tricene-SDS-PAGE gels (Shägger & von Jagow 1987) run overnight at 100v constant to prepare samples for amino acid sequence analysis. A range of molecular mass standards was included in each run: Lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), bovine carbonic anhydrase (30 kDa), ovalbumin (45 kDa), BSA (66.2 kDa), phosphorylase B (97.4 kDa). Protein bands were visualised by staining with Coomassie blue or by silver staining.

2.1.6 Electroblotting

Proteins in SDS-PAGE gels were transferred to nitro-cellulose membranes (Schleicher and Scheull, Dassel, Germany) or PVDF membrane (Immobilon P, Millipore U.K. Ltd) using a semi-dry transfer apparatus (Kyhse-Andersen, 1984) with a current of 1.0 mA/cm^2 of gel for 1 hour. After transfer, nitro-cellulose membranes were incubated in a solution of 0.1% w/v Tween 20 (Sigma) in PBS pH 7.5

(PBS/T20) for 30 min at room temperature to block non-specific protein adsorption to the membranes. Following the blocking procedure the membranes were rinsed in fresh PBS/T20 (all subsequent washes and antibody dilutions were in PBS/T20) and transferred to optimally diluted sheep anti-MMCP-1, rabbit anti-MMCP-1 or rabbit anti-RMCP I for 1 h at room temperature. The membranes were subsequently washed (3 x 5 min) and probed with optimally diluted pig anti-sheep IgG-horseradish peroxidase (HRPO) conjugate prepared according to the method of Nakane & Kawaoi (1974) or sheep anti-rabbit IgG-HRPO conjugate (Sera Lab., Crawley, Sussex). Peroxidase activity was revealed with 3-3'-diaminobenzidine/H_2O_2 (Graham & Karnovsky, 1966). PVDF membranes with protein bands for sequence analysis were stained with Coomassie blue, immediately after transfer was completed, and de-stained in methanol.

2.1.7 Deglycosylation

Aliquots of the proteases in distilled water (20-25 µl), containing approximately 2 µg of protein, were denatured by heating in a boiling water bath for 10 min and then cooled to room temperature. Denatured samples were incubated with 10 µl of deglycosylation buffer (150 mM phosphate buffer pH 7.0, 50 mM EDTA and 1% v/v 2-mercaptoethanol) and 1 µl (0.2 units) of peptide-N-glycosidase F (PNGase F) or endo-a-acetyl-galactosaminidase; (both from Boehringer-Mannheim, Lewes, UK) at 37°C overnight. An additional 0.2 units of glycosidase were then added and incubation was continued for a further 24h.

2.1.8 Enzyme linked immunosorbent assay to quantify MMCP-1.

Mouse mast cell proteases were quantified by an antigen capture ELISA described previously (Huntley *et al.*, 1990a) except that the capture antibody was affinity-purified sheep anti-MMCP-1 which was diluted to 1 $\mu g.ml^{-1}$ for coating ELISA plates.

2.1.8.1 Protocol

Plate coating:-

Plates were coated with a solution of sheep anti-MMCP-1 capture antibody diluted to $1\mu g.ml^{-1}$ with 0.1 M carbonate buffer pH 9.6. 50 µl of diluted antibody was loaded into each well and the plate tapped gently to ensure even distribution of the antibody solution. The coated plates were incubated in a humid chamber at +4°C for 24h before use.

2.1.8.2 ELISA test.

1. Wash coated plates x6 with phosphate buffered saline (PBS) + 0.05%^{V}/v tween 20 (Sigma).
2. Load standards (see below) and unknown sample (50 µl/well).
3. Incubate for 1.5h at room temperature. (All dilutions in 4% BSA in PBS/Tween 20).
4. Wash plates x6 with PBS/Tween 20.
5. Incubate plates with conjugate, optimally diluted with 4% BSA in PBS/Tween 20, (50 µl/well) at room temperature for 1h.

6) Wash plates x6 with PBS/Tween 20.

7) Incubate plates with OPD/H_2O_2 substrate (50 µl/well) for 20-25 min.

8) Stop reaction with 2.5M H_2SO_4 (25µl/well).

9) Read plates at 492 nm.

2.1.8.3 Preparation of standards.

MMCP-1 in serum:-

Stock MMCP-1 was diluted to 50 µg.ml^{-1} with normal mouse serum. This solution was further diluted to 20 ng.ml^{-1} with PBS/Tween 20/BSA and used to make up working standards, 0.25, 0.5, 1, 2, 4, 8, 10, 12 ng/ml. Standards were run in duplicate on each plate.

MMCP-1 in tissue extracts:-

Stock MMCP-1 was diluted to 20 ng.ml^{-1} with PBS/Tween 20/BSA and subsequently diluted as for serum standards.

Volumes required to make up 100µl of standard

Conc. required (ng.ml^{-1})	0.25	0.5	1.0	2.0	4.0	8.0	10.0	12.0
Vol. 20 ng.ml^{-1} stock (µl)	1.25	2.5	5.0	10.0	20.0	40.0	50.0	60.0
Vol. Diluent (µl)	98.75	97.5	95.0	90.0	80.0	60.0	50.0	40.0

2.1.8.4 Buffer solutions for ELISA.

1) 0.1 M carbonate buffer pH 9.6

0.1 M $NaCO_3$; 0.1 M $NaHCO_3$ titrated to pH 9.6

2) Citrate/phosphate buffer pH 5.0

Stock solutions:-

0.1 M citric acid

0.2 M Na_2HPO_4

Working buffer:-

0.1M citric acid	24.3 ml
Na_2HPO_4	25.7 ml
distilled water	50 ml

3) Substrate:-

Citrate/phosphate buffer pH 5.0	100 ml
Ortho phenylene diamine (Sigma)	40 mg
H_2O_2	40 µl

2.1.9 Histochemistry and Immunohistochemistry.

Tissue sections were stained with toluidine blue pH 0.5 (Enerbäck, 1966b) or with sheep anti-MMCP-1, directly conjugated to FITC (Harlow & Lane, 1988), by the following method; re-hydrated sections (5 µm thick) were incubated in 5% w/v BSA (Sigma, Grade IV) in PBS for 30 min before transfer to sheep anti-MMCP-1-FITC, optimally diluted in 5% BSA/PBS for 1 hour at room temperature. After

washing (3 x 5 min in PBS) sections were mounted in citifluor non-fluorescent mountant (Citifluor Ltd. London, UK.)

2.1.10 Specific anti-protease antibodies

Rabbit antiserum to MMCP-1 was prepared as described previously (Newlands *et al.*, 1987). Rabbit anti-RMCP I antibodies were raised, absorbed against MMCP-1-sepharose and affinity purified on RMCP I-sepharose as described previously (Miller *et al.*, 1988). Antiserum to MMCP-1 was raised in sheep by intramuscular injection of 100 μg MMCP-1 in Freund's complete adjuvant. Two subsequent injections of MMCP-1 in Freund's incomplete adjuvant were given 4 and 7 weeks after the first. The sheep was bled at 2 week intervals after the final injection and batches of serum with an antibody titre > 1/16 by double diffusion against MMCP-1 were applied to an MMCP-1 sepharose affinity column and eluted anti-MMCP-1 antibodies were cross absorbed against RMCP I as described for rabbit anti-MMCP 1 (Miller *et al* 1988).

2.1.11 Binding of [^{3}H] diisopropylfluorophosphate ([^{3}H]-DFP).

Protease samples (40 μl) containing 4-8 μg of protein were incubated with 10 μ Ci [^{3}H]-DFP (5.8 Ci/mMol; Amersham International plc., UK) at 37°C for 15 min. The samples were prepared for electrophoresis by the addition of 50 μl of reducing sample buffer (Laemmli, 1970) and heated to > 90°C for 3 min. 5 μl aliquots of sample were loaded to each lane of the gel. After electrophoresis the gels were prepared for auto-radiography by the method of Laskey & Mills (1975). Radiographs were exposed at -70°C and developed after 21 days.

2.1.12 Specific activities of enzyme preparations.

Specific activity was determined by measuring the rate of hydrolysis of the synthetic substrate, CBZ-L-tyrosine 4-nitrophenyl ester (CBZ-L-tyr-4NPE). The assay was performed as follows;

20 mM tris/HCl, pH 7.8	195 µl
4 mM CBZ-L-tyr-4NPE in DMSO	5 µl
Enzyme preparation	5 µl

Incubate at 22°C for 5 min and read at 405 nm.

Specific activity was calculated from the initial rates of substrate hydrolysis as follows.

Equation 1. Calculation of specific enzyme activity.

$$\frac{\Delta A_{405}/_{\text{second}} \times \text{volume of reaction mixture in litres}}{1.88^4 \times \text{mg of enzyme in reaction mixture}}$$

The molar extinction coefficient of nitrophenol was taken as 1.88^4/l/mole/cm at 405 nm and pH 7.8.

Enzyme activity is expressed in nkat/mg of protein; 1 nkat is that activity which hydrolyses 1 nMol of substrate in 1 second. All activities were corrected for spontaneous alkaline hydrolysis of the substrate by subtraction of a parallel blank which contained no enzyme.

2.1.13 Substrate Kinetics.

All kinetic measurements were made using a Beckman DU 600 spectrophotometer. Rates of hydrolysis of 5 concentrations (0.125 to 2 mM) of the chymotryptic substrate Succ-Ala-Ala-Pro-Phe-4NA were measured by adding 2 µl of substrate solution to 48 µl of enzyme solution in 0.1M tris/HCl pH 7.5. The increase in absorbance was continuously measured at 410 nm and the spectrophotometer's on-board software used to calculate the kinetic constants K_m and k_{cat} from the initial rates of substrate hydrolysis.

2.1.14 Active site titration

Concentrations of protease were determined by active-site titration of the MMCP-1-like proteases against the fluorogenic substrate 4-methyl umbelliferyl-p-(NNN trimethylammonium) cinnamate; (MUTMAC; Sigma, Poole, UK; Jameson, Roberts, Adams, Kyle & Elmore, 1973). Fluorescence was measured on a Perkin Elmer 3000 fluorescence spectrophotometer with an excitation wavelength of 365 nm and an emission wavelength of 445 nm. Unknown samples were measured, in triplicate, against a standard curve prepared from 4-methyl-umbelliferone (7-hydroxy-4-methylcoumarin; Aldrich Chemical Co. Ltd.).

The assay conditions were as follows;

MUTMAC	40 µl
Methanol	20 µl
Enzyme	5 µl
Buffer	1935 µl

The buffer was 8.695 mM KH_2PO_4, 30.43 mM Na_2HPO_4, pH 7.41. The MUTMAC, and enzyme were mixed and incubated at room temperature for 5, 10, 15 or 20 min before addition of buffer and methanol and read on the spectrofluorimeter with an excitation wavelength of 365 nm and an emission wavelength of 445 nm. Initial tests showed that maximal fluorescence was obtained after 15 to 20 min and therefore all subsequent incubations were for 20 min before reading. The standard curve was prepared from the following range of standard dilutions of 4-methyl-umbelliferone; 0.25, 0.5, 1.0, 2.0, 3.0 and 6.0 µM in methanol from a 10 µM stock.

4-Methyl-umbelliferone standard.	20 µl
phosphate buffer	1980 µl

These were also read on the spectrofluorimeter with an excitation wavelength of 365 nm and an emission wavelength of 445 nm. The standard curve was linear over the whole range tested and linear regression analysis was therefore used to calculate the concentrations of unknown samples from the regression equation.

2.1.15 Inhibitor sensitivity

2.1.15.1 Synthetic inhibitors.

Sensitivity of isolated enzymes to the following specific protease inhibitors was studied using CBZ-L-Tyr-4NPE as substrate; 2.0 mM phenyl-methyl-sulphonyl-fluoride (PMSF), 20 mM 3,4-Dichloro-isocoumarin (3,4 DCI), 200 mM N-[N-(L-3-Trans-carboxyoxirane-2 carbonyl)-L-leucyl] -agmatine (E64), 2 mM 1,10-phenanthroline (1,10-Phe), 1 mM pepstatin. (All reagents supplied by Sigma, Poole, U.K.). The assay of enzyme activity was as follows; 2.5 µl of enzyme preparation and 2.5 µl of inhibitor were pre-incubated with 40 µl of 0.1M tris/HCl buffer pH 7.5 at 22°C for 1 h. The enzyme concentration in the incubation mixture was 50 nM and the inhibitor concentrations were those given above. After pre-incubation 5 µl of substrate was added and incubated for a further 3 min. Enzyme activity was quenched by the addition of 450 µl of 70% $^{v}/_{v}$ methanol. Absorbance was measured at 405 nm and percentage inhibition calculated in comparison with an enzyme preparation incubated without inhibitor.

2.1.15.2 Inhibition kinetics with α_1-proteinase inhibitor.

The association constant (k_{ass}) for the reaction between the MMCP-1-like proteases and human α_1-proteinase inhibitor (α_1-PI; Sigma) was determined by pre-incubating equimolar enzyme and α_1-PI for periods of 15 to 120 s before continuous measuring the rate of hydrolysis of the substrate Succ-Ala-Ala-Pro-Phe-4NA. The k_{ass} for each reaction was calculated by non-linear regression as described previously

(Pirie-Shepherd, Miller & Ryle; 1991). The inhibition constant (K_i) for the reaction between each protease and α_1-PI was determined by incubating protease with varying inhibitor concentrations (0.25 to 1.75 molar equivalents) for 35 times $t_{1/2}$ (the time to half inhibition for an equimolar reaction calculated from the equation $t_{1/2} = 1/k_{ass}.E_0$ where E_0 is the initial enzyme concentration). The apparent K_i (K_i^{app}) was calculated by non-linear regression, as described previously (Pirie-Shepherd *et al* 1991) and the true K_i calculated from the equation $K_i = K_i^{app} /1+S_0/K_m$, where S_0 is the initial substrate concentration.

2.1.16 N-terminal sequence analysis.

Amino terminal sequence analyses of MMCP-1A-E were performed on soluble proteins in 50 mM MES directly from ion-exchange on an Applied Biosystems 477A protein microsequencer by the Welmet protein characterisation facility, Biochemistry Department, University of Edinburgh. MSMCP, immobilised on Immobilon P following tricene-SDS-PAGE and Western blotting was sequenced by the Microchemical facility, AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge, UK.

2.2 The role of stem cell factor in regulating mast cell growth and function in normal, or parasitised rats.

2.2.1 Animals

Random-bred female Wistar rats weighing 250 to 300 gm, and maintained under conventional conditions at Moredun Research Institute, were used in all experiments to study the role of SCF in regulating mast cell growth and function.

2.2.2 Parasites

Rats were infected with 2500-3000 *N. brasiliensis* third stage larvae by sub-cutaneous injection (Huntley *et al,* 1993) or with 750-1000 *T. spiralis* muscle larvae *per os* (Woodbury *et al,* 1984). In both of these procedures the rats were anaesthetised with halothane (May and Baker, Dagenham, Essex, U.K.). The *T. spiralis* muscle larvae were kindly provided by Mr. H. Urquhart, Centre for Tropical Veterinary Medicine, University of Edinburgh.

2.2.3 Intraperitoneal treatment of rats with Stem Cell Factor and/or Lymph Node Conditioned Medium.

2.2.3.1 Preparation of Lymph Node Conditioned Medium (LNCM).

LNCM was prepared from mesenteric lymph node cells of normal rats after the method of Haig, *et al* (1988). Aliquots (200 ml) of 4×10^6 per ml viable nucleated MLN cells were prepared in Iscove's serum-free medium, which contained 5×10^{-5} M 2-mercaptoethanol (2-ME), 100 U/ml penicillin, 50 $\mu g.ml^{-1}$ streptomycin, 100 $\mu g.ml^{-1}$ bovine serum albumin (BSA; Sigma, Poole, U.K.), 80 $\mu g.ml^{-1}$ phosphatidyl choline (Sigma), 25 $\mu g.ml^{-1}$ transferrin (Sigma) and 2 $\mu g.ml^{-1}$ concanavalin A (Con A; Miles, Slough, U.K.). Cultures were incubated for 48 hours at 37°C in 120 cm^2 tissue culture flasks (Nunc, Paisley, U.K.) in an atmosphere of 5% CO_2. LNCM was harvested by centrifugation and sterilised by filtration through a 0.22 μm filter (Millipore, Harrow, U.K.) prior to storage at -70°C. Batches of LNCM and dilutions of them were tested for their ability to generate mast cells from Wistar rat bone marrow and were used in the experiments described below at the optimal concentration (20 - 50%).

2.2.3.2 Treatment of rats with LNCM or SCF.

Female Wistar rats, 10 months old were used in these experiments. Groups of 4 rats were injected intraperitoneally with;

1. 0.5 ml LNCM diluted to 1 ml with serum-free IMDM;
2. 0.5 ml LNCM plus 320 µl pegylated recombinant rat stem cell factor, from a bacterial expression system ($rrSCF^{164}$; 10 $\mu g.ml^{-1}$) diluted to 1 ml with serum-free IMDM;
3. 320 µl SCF (10 $\mu g.ml^{-1}$) diluted to 1 ml with serum-free IMDM;
4. 1.0 ml serum-free IMDM;
5. 1.0 ml serum-free IMDM containing 3 $\mu g.ml^{-1}$ Concanavalin A, the same initial concentration as was present in the LNCM.

The rats in groups 1 - 3 were injected daily for 2, 4 or 7 consecutive days and those in groups 4 and 5 were injected for 7 consecutive days. Inoculations were carried out under halothane anaesthesia and the rats were killed by overdose of halothane/CO_2 followed by cervical dislocation. Peritoneal cells were harvested by lavage of the peritoneal cavity with 15 ml of HBSS containing 1% w/v BSA. The peritoneal cells were washed with HBSS/BSA and cyto-centrifuge preparations were made and either fixed 4% Paraformaldehyde in PBS for 1h at 45°C followed by 70% ethanol for 1h or over night as required or stained with Leishman's stain by the following procedure. Slides were prepared in the cyto-centrifuge and allowed to air-dry for 5 min. 1 ml of undiluted Leishman's stain (BDH, Poole, U.K.) was applied to the slide for 3 min. After this initial staining period the stain was diluted with 3 ml of tap water and the slide was stained for a further 9 min. The slides were subsequently

washed in tap water to remove excess stain and allowed to air-dry. Dried, stained slides were cleared by immersion in xylene and mounted with a synthetic resin mountant before examination with a Leitz Dialux microscope (Leitz, Wetzlar, Germany).

2.2.3.3 Paired Immunofluorescence for detection of RMCP I and RMCP II in cyto-centrifuge preparations.

1. Re-hydrate cyto-centrifuge preparations.
2. Incubate sections in diaminobenzidine (4 mg/10 ml in Phosphate buffered saline (PBS) + 50 µl H_2O_2) for 15 min. to quench endogenous fluorescence of eosinophils. Wash well in distilled water.
3. Incubate sections in 5% $^{W}/_{v}$ Bovine serum albumin (BSA) in PBS for 30 min.
4. Incubate sections in affinity-purified rabbit anti-RMCP I, cross-absorbed against RMCP II and diluted to approximately 2 µg/ml for 1h at room temperature.
5. Wash 3 x 5 min in PBS.
6. Incubate sections with optimally diluted sheep anti-rabbit-tetra-methylrhodamine isothiocyanate (TRITC) conjugate *.
7. Wash 3 x 5 min. in PBS.
8. Incubate sections with monoclonal mouse anti-RMCP II, developed at Moredun Research Institute (Huntley *et al* 1990b) diluted to approximately 2µg/ml for 1h at room temperature.
9. Wash 3 x 5 min. in PBS.
10. Incubate with optimally diluted sheep anti-mouse IgG-fluorescein isothiocyanate (FITC) conjugate containing 2% normal rabbit serum *.

11.Wash 3 x 5 min. in PBS and mount in non-fluorescent mountant.

Dilutions.

All primary antibody and conjugate dilutions were made in 5% BSA in PBS.

* conjugates were centrifuged well to remove debris.

2.2.4 Intravenous treatment of rats with Stem Cell Factor

Recombinant rat stem cell factor[164] (rrSCF[164]: Amgen Inc. Thousand Oaks ca. U.S.A.) modified by the covalent attachment of polyethylene glycol (Tsai *et al,* 1991) was administered intravenously to anaesthetised rats for 14 consecutive days in 1 ml physiological saline at the rate of 25μg/kg of body weight/day, control rats were sham treated with physiological saline alone. These inoculations were carried out by Prof. H.R.P. Miller of the University of Edinburgh.

2.2.5 Antibodies to SCF.

Polyclonal anti-rrSCF[164] antibodies were produced by inoculating a sheep with 100 μg of rrSCF[164], suspended in 4 ml of Freund's complete adjuvant intramuscularly, into both hind legs. This procedure was repeated 4 and 8 weeks after the initial injection except that Freund's incomplete adjuvant was used for the subsequent inoculations. Blood samples were taken 3 weeks after the final inoculation and the serum tested by ELISA for anti-rrSCF[164] activity. Immune sera that gave absorbances more than five-fold greater than the background absorbance of pre-immunisation serum when tested at dilutions of up to 1/80,000 by ELISA were used to prepare specific antibody. The anti-SCF antibody preparation virtually abolished the effects of

rrSCF^{164} on the survival and proliferation of *in vitro*-derived rat mast cells, but had no detectable effect on the IL-3 dependent proliferation of these cells (MacDonald, Thornton, Newlands, Galli, Moqbel & Miller, 1996).

An immuno-affinity column was prepared by coupling 1.6 mg of rrSCF^{164} to CNBr-activated Sepharose-4B (Pharmacia Biotech, St. Albans, Herts. U.K.) in accordance with the manufacturer's instructions. Aliquots of serum (5 ml) were applied to the column, which had been equilibrated with phosphate buffered saline (PBS) and the column was subsequently eluted with 0.1M citric acid pH 2.2 + 0.5M NaCl to recover bound, specific antibody. For control purposes, sheep immunoglobulin was prepared by precipitation of normal sheep serum with 50% saturated ammonium sulphate followed by gel filtration chromatography of the re-dissolved precipitate on Sephacryl S-200 (Pharmacia Biotech). Immunoglobulin preparations were concentrated by vacuum dialysis in a collodion thimble apparatus (Sartorious, Epsom, Surrey, U.K.) against PBS.

2.2.6 Treatment with anti-SCF antibodies

Rats were inoculated intraperitoneally with 1 mg sheep anti-rrSCF^{164} or normal sheep IgG in 1 ml phosphate buffered saline (PBS). Normal rats were treated daily for each of 4 or 7 days to determine the effects on resting mast cell populations. Because of the tissue and pulmonary migration of the L_3 and L_4 larval stages of *N. brasiliensis*, rats infected with this parasite were treated on day 3 of infection when the L_4 larvae first reached the intestine and on days 5, 7, 10 and 12 thereafter. A further experiment was carried out with *N. brasiliensis*-infected rats to determine whether treatment with anti-SCF had an effect on mast cell populations already expanded in response to

parasitic infection. In this experiment rats were treated with anti-SCF daily on days 10 to 13 after infection. For rats infected with *T. spiralis*, treatment commenced on day 0 and continued on days 3, 5, 7 and 10. Normal rats were killed 24 hours and parasitised rats 24 or 48 hours after the final inoculation by exsanguination under deep halothane anaesthesia followed by cervical dislocation.

2.2.7 Haematological studies

Blood samples were obtained by tail-snip under halothane anaesthesia with the blood collected into heparinised tubes. Total red cell and leukocyte counts were performed on a model ZM Coulter counter, haemoglobin concentrations were measured on a Coulter haemoglobinometer and packed cell volume by micro-haematocrit. Blood films were stained with Leishman's stain (2.1.3) for differential cell counts in which 100 leukocytes were counted per blood film.

2.2.8 Material collected post mortem

Peritoneal cells were collected by lavage of the peritoneal cavity with 20 ml of PBS containing 0.1% $^{w}/_{v}$ BSA (PBS/BSA). Small intestine (mid-jejunum), stomach, lung, liver and tongue were also collected for analysis and the tissues were either fixed by immersion in 4% $^{w}/_{v}$ paraformaldehyde in PBS for 6 hours followed by 70% $^{v}/_{v}$ ethanol overnight (Newlands *et al*, 1987) before processing to paraffin wax or stored frozen at -20°C before further processing. Serum was also stored at -20°C. Peritoneal lavage samples were sedimented by centrifugation at 1000 xg for 20 min at +4°C. The cells were re-suspended in 1 ml PBS/BSA, re-centrifuged at 1000 xg for 5 min and finally re-suspended in 1 ml PBS/BSA for estimation of mast cell numbers. Mast cell

counts were performed by diluting a 10 µl aliquot of the cell suspension with 90 µl of a mast cell stain containing 0.5% $^{w}/_{v}$ methylene blue in 50% $^{v}/v$ propylene glycol. Blue staining mast cells were counted using an Improved Neubauer haemocytometer.

Concentrations of Rat mast cell proteases I and II (RMCP I and II) were measured in serum and tissue homogenates by Enzyme Linked Immunosorbent Assays (ELISA).

2.2.9 RMCP I ELISA PROTOCOL

2.2.9.1 Plate coating:-

Coat plates with a solution of capture antibody diluted to 1µg/ml in 0.1 M carbonate buffer pH 9.6 load 50 µl/well

2.2.9.2 Sample preparation:-

Tissue homogenates prepared in 1.5M NaCl buffered with 20 mM Tris pH 7.5. Subsequent sample and standard dilutions in 4% BSA in PBS/0.05% Tween 20 + 0.5 M NaCl.

2.2.9.3 ELISA test.

1) Wash coated plates x6 with PBS/Tween 20

2) Load standards and samples (50 µl/well) and incubate for 1h at room temperature.

3) Wash x6 with PBS/Tween 20

4) Incubate plates with rabbit anti-RMCP I (50 µl/well) for 1h at room temp.

5) Wash x6 with PBS/Tween 20

6) Incubate plates with optimally diluted anti-rabbit-HRPO conjugate (50 µl/well) 1h at room temp.

7) Wash x6 with PBS/Tween 20

8) Incubate plates with OPD/H_2O_2 substrate.(50 µl/well)

9) Stop reaction with 25 µl 2.5 M H_2SO_4

10) Read plates at 492 nm.

2.2.9.4 Standards

Dilute stock RMCP I initially to 1µg/ml and then to 20 ng/ml and use this solution to prepare a range of standard dilutions (0.5, 1, 2, 4, 6, 8, 10, 12 ng/ml)

Volumes required to make up 100µl of standard (ml)

Conc. required (ng/ml)	0.5	1.0	2.0	4.0	6.0	8.0	10.0	12.0
Vol. 20 ng/ml stock (µl)	2.5	5.0	10.0	20.0	30.0	40.0	50.0	60.0
Vol. Diluent (µl)	97.5	95.0	90.0	80.0	70.0	60.0	50.0	40.0

2.2.9.5 Solutions

1) 0.1 M carbonate buffer pH 9.6

0.1 M $NaCO_3$; 0.1 M $NaHCO_3$ titrated to pH 9.6

2) Citrate/phosphate buffer pH 5.0

2.2.9.6 Stock solutions:-

0.1 M citric acid

0.2 M Na_2HPO_4

Working buffer:- add 24.3 ml 0.1 M citric acid, 25.7 ml 0.2 M Na_2HPO_4 and 50 ml distilled water

3) Substrate:-

Citrate/phosphate buffer pH 5.0	100 ml
Ortho phenylene diamine (Sigma)	40 mg
H_2O_2	40 µl

4) Diluent for samples and standards:-

Phosphate buffered saline containing 4%^{W}/v Bovine serum albumin (Sigma), 0.05%^{V}/v Tween 20 (Sigma) and 0.5 M NaCl.

5) Diluent for antibody reagents:-

Phosphate buffered saline containing 4%^{W}/v Bovine serum albumin , 0.05%^{V}/v Tween 20.

6) Wash buffer:-

Phosphate buffered saline containing 0.05% Tween 20

2.2.10 RMCP II ELISA Protocol

2.2.10.1 Plate coating:-

Coat plates with a solution of monoclonal antibody diluted to 1 μg/ml with 0.1M carbonate buffer pH 9.6. Load 50μl of diluted antibody into each well and tap the plate gently to ensure even distribution. Incubate the coated plates in a humid chamber at +4°C for 24h before use (coated plates can be stored at +4°C for several days before use).

2.2.10.2 ELISA test.

1) Wash coated plates x6 with phosphate buffered saline (PBS) + 0.05%^{V}/v tween 20 (Sigma).

2) Incubate plates with 4% ^{W}/v Bovine serum albumin (BSA; Sigma) in PBS/Tween 20 for 30 min at room temperature.

3) Wash plates x1 with PBS/Tween 20 and load standards (see below) and unknown sample (50μl/well). Incubate for 1.5h at room temperature (all dilutions in 4% BSA in PBS/Tween 20).

4) Wash plates x6 with PBS/Tween 20.

5) Incubate plates with conjugate optimally diluted with 4% BSA in PBS/Tween 20 (50μl/well) at room temperature for 1h.

6) Wash plates x6 with PBS/Tween 20.

7) Incubate plates with OPD/H_2O_2 substrate (50μl/well).

8) Stop reaction with 2.5M H_2SO_4 (25μl/well).

9) Read plates at 492 nm.

2.2.10.3 Standards

2.2.10.3.1 RMCP II in serum:-

Dilute stock RMCP II to 2μg/ml with normal rat serum. Further dilute this 2 μg/ml stock to 20 ng/ml with PBS/Tween 20/BSA and use this to make up working standards, 0.25, 0.5, 1, 2, 4, 8, 10, 12 ng/ml. Standards are run in duplicate on each plate.

2.2.10.3.2 RMCP II in tissue extracts:-

Dilute stock RMCP II to 2μg/ml with PBS/Tween 20/BSA then proceed as for serum standards.

Volumes required to make up 100μl of standard (μl)

Conc. required (ng/ml)	0.5	1.0	2.0	4.0	6.0	8.0	10.0	12.0
Vol. 20 ng/ml stock (μl)	2.5	5.0	10.0	20.0	30.0	40.0	50.0	60.0
Vol. diluent (μl)	97.5	95.0	90.0	80.0	70.0	60.0	50.0	40.0

2.2.10.4 Solutions.

1) 0.1 M carbonate buffer pH 9.6

0.1 M Na_2CO_3; 0.1M $NaHCO_3$ titrated to pH 9.6

2) Citrate/phosphate buffer pH 5.0

2.2.10.5 Stock solutions:-

0.1 M citric acid

0.2 M Na_2HPO_4

Working buffer:-

add 24.3 ml 0.1 M citric acid, 25.7 ml 0.2 M Na_2HPO_4 and 50 ml distilled water.

3) Substrate:-

Citrate/phosphate buffer pH 5.0	100 ml
Ortho phenylene diamine (Sigma)	40 mg
H_2O_2	40µl

2.2.11 Assessment of rat anti-sheep antibodies

Because ruminant proteins are likely to have been included in the rats' diet and the experimental rats may mount an immune response against sheep proteins, an ELISA was developed to quantify the antibody response against sheep immunoglobulins. ELISA plates (Dynatech M129B) were coated with a solution of normal sheep immunoglobulin, prepared as described above, in 0.1 M carbonate buffer pH 9.6 (50 µl/well). The plate was incubated at +4°C overnight and then washed x6 with PBS containing 0.05% V/v Tween 20 (Sigma, Poole, Dorset, U.K.) before loading with samples. Sera from the animals which had been treated with anti-rrSCF[164] or normal sheep IgG and pre-treatment serum samples from the same rats was prepared in serial dilutions in PBS and loaded, in triplicate, onto the plate (50 µ l/well) and then incubated at room temperature for 1 hour. The ELISA plate was washed x6 with PBS/Tween 20 and a sheep anti-rat IgG-horse radish peroxidase

conjugate (Sigma), optimally diluted with PBS/Tween 20, was applied. The plate was again incubated at room temperature for 1 hour, washed x6 as before and the colour reaction developed, using ortho-phenylenediamine (0.4 $mg.ml^{-1}$ in citrate/phosphate buffer, pH 5.0, at the rate of 50 µl/well) as substrate, for 10 min and the reaction was terminated by the addition of 25 µl of 2.5 M H_2SO_4. The plate was read on a Titertek Multiskan MC ELISA plate reader (Titertek, Paisley U.K.) at 492 nm. Samples which had absorbances which were significantly higher, by Student's t-test, than the pre-treatment control samples were considered to be positive.

2.2.11.1 Histological assessment.

Histological sections (5 µm thick) were stained with Toluidine blue (0.5% w/v, pH 0.5) and mast cells in the jejunal mucosal epithelium and lamina propria were enumerated on a Leitz Dialux microscope with a x25 objective lens and x12.5 eyepieces equipped with a 100 mm^2 graticule. A minimum of 5 fields were counted per section.

2.2.12 Data presentation and analysis

Unless otherwise specified, all data are presented as the mean ± s.e.m. The 2-tailed Students t-test was used to analyse data that were normally distributed, whereas the Mann-Whitney U test was used as the non-parametric test. The time course of responses in different groups of rats were examined for statistical significance by using analysis of variance (ANOVA). $p < 0.05$ was taken to indicate a significant difference.

2.3 Studies on the role of SCF in mast cell regulation in *S. mansoni*-infected rats.

2.3.1 Animals.

Fischer F344 inbred female rats, purchased from Harlan Olac (Bicester Oxon.), 6 to 8 weeks old and weighing approximately 150g, were used in this experiment. The rats were maintained under standard conditions at the Department of Biology, University of York, with water and pelleted diet freely available.

2.3.2 Parasites.

Infection with approx. 2500 *S. mansoni* cercariae via shaved abdominal skin was by the ring technique (Smithers & Terry 1965), carried out under anaesthesia with optimal doses of Hypnorm/Hypnovel administered by intra-peritoneal injection.

2.3.3 Blood samples.

Blood samples were taken on days 18, 21, 24, 27, and 30 after infection by tail-snip. Approx. 300 µl of blood was obtained from each rat under halothane anaesthesia and allowed to clot at room temperature. Serum was harvested and stored at -20°C.

2.3.4 Anti-SCF antibody treatment.

The rats were treated with 1 mg sheep anti-SCF or normal sheep IgG on days 21, 24, 27 and 30 by intraperitoneal injection. An interval of at least 2h was allowed between blood-sampling and administration of antibody.

2.3.5 Material collected post-mortem.

On day 32 the rats were anaesthetised by intraperitoneal injection of Hypnorm/Hypnovel and blood samples taken as before. The hepatic portal system was

perfused to recover adult worms (Smithers & Terry 1965). The liver was excised and a portion weighed prior to storage at -20°C. Slices were cut from the remainder and immersed in 4% paraformaldehyde for 6 h and subsequently in 70% ethanol (Newlands *et al* 1987). Similarly, a portion of jejunum was also excised, flushed through with PBS and divided into two parts for storage at -20°C or fixation. These procedures were carried out by Prof. R.A. Wilson and Dr. P.S. Coulson at the University of York. Fixed tissues were subsequently processed and embedded in paraffin wax before sectioning and staining with either toluidine blue for the demonstration of mast cells or carbol chromotrope for eosinophils. Rat mast cell proteases I and II were measured in serum and tissue extracts by antibody capture ELISA as described previously (Huntley *et al* 1990). Cell counts were performed as described previously (Miller *et al* 1994) and the results expressed as cells per 0.2 mm^2. Data are presented as mean ± sem and were analysed by Student's two sample t-test or, for time course data by analysis of variance (ANOVA) using the Minitab statistics programme.

2.4 Development of an ELISA test to measure SCF in the sera of normal or parasitised rats.

2.4.1 Animals

Random-bred female Wistar rats weighing 250 to 300 gm, and maintained under conventional conditions at Moredun Research Institute, were used in all studies.

2.4.2 Parasites.

Infection of rats with *N. brasiliensis* or *T. spiralis* was as described in section 2.2.2.

2.4.3 Stem Cell Factor.

Treatment of rats with rrSCF^{164} was as described in section 2.2.4.

The total blood volume of rats weighing approximately 250g has been estimated at 50 ml for each kg of bodyweight and the total plasma volume at 30 ml for each kg of bodyweight (Schalm, Jain & Caroll, 1975); thus the SCF dose/ml/day can be calculated from the following equation.

Equation 2. Calculation of SCF concentration in serum.

$$\frac{\text{Dose } (\mu g/kg \text{ bodyweight}) \times \text{bodyweight (kg)}}{\text{Serum volume (ml/kg bodyweight)} \times \text{bodyweight (kg)}} = \frac{25 \times 0.25}{30 \times 0.25} = 0.833\ \mu g.ml^{-1} \text{ of serum}$$

2.4.4 Antibodies

Polyclonal antibodies to rrSCF^{164} were produced and purified by immuno-affinity chromatography as described previously (Section 2.2.5). For control purposes, sheep immunoglobulin was prepared by precipitation of normal sheep serum with 50% saturated ammonium sulphate also as described (Newlands, Miller, MacKellar & Galli, 1995). Biotinylation of affinity purified sheep anti-SCF was by the technique described by Goding (1983).

2.4.5 Treatment with anti-SCF antibodies

Treatment with polyclonal antibodies was as described in section 2.2.6.

2.4.6 Optimisation of SCF ELISA

The format of the ELISA was that of antigen capture with polyclonal anti-SCF and of detection of captured SCF with the same antibody which had been biotinylated, followed by streptavidin-HRPO (Boehringer-Mannheim, U.K.).

The capture antibody, affinity purified polyclonal sheep anti-SCF, was coated onto the test plates (Dynatech M129B) at the rate of 0.1, 0.5, 1.0, 5.0 or 10.0 $\mu g.ml^{-1}$ in 0.1M carbonate buffer pH 9.6.

The optimum dilution of the biotinylated, sheep anti-SCF, secondary antibody for the standard concentration range, was determined by titration at 1/125, 1/250, 1/500 and 1/1000 of stock antibody which had a concentration of 1 $mg.ml^{-1}$. Final detection in the titration of both capture and secondary antibodies was with streptavidin-HRPO, standardised at a dilution of 1/500 in accordance with the manufacturers recommendation, and utilising orthophenylenediamine (Sigma, Poole, UK) at a concentration of 0.4 $mg.ml^{-1}$ and H_2O_2 (60 µl/10 ml) as substrate. Plates were read at 492 nm on a Titertek MC ELISA plate reader (Titertek-Flow, Paisley, UK).

Samples and standards were diluted 1:20 with 5% $^{w}/_{v}$ bovine serum albumin (Sigma) in phosphate buffered saline (BSA/PBS). Standard curves were prepared which covered the range 0.5 pg to 20 $ng.ml^{-1}$. In order to determine whether the assay would detect physiological concentrations of SCF in serum, normal rat serum was spiked with $rrSCF^{164}$ at a concentration of 1$\mu g.ml^{-1}$. A range of dilutions of this spiked serum, in the range 5 to 750 $pg.ml^{-1}$ were prepared in BSA/PBS for assay.
The different coating antibody densities gave a concentration dependent increase in absorbance at 492 nm up to 1.0 $\mu g.ml^{-1}$, with a standard curve which covered the range 5 to 750 $pg.ml^{-1}$, and did not increase significantly at higher concentrations. Further optimisation of the test was therefore based on plates coated at the rate of 1.0 $\mu g.ml^{-1}$.

Dilutions of biotinylated sheep anti-SCF at 1/125, 1/250 and 1/500 gave very similar results, with somewhat lower absorbances for the 1/1000 dilution. A 1/500 dilution of the biotinylated, sheep anti-SCF, secondary antibody was therefore used in later tests.

2.4.7 Data presentation and analysis

Unless otherwise specified, all data are presented as the mean ± s.e.m. The 2-tailed Student's t-test was used to analyse data that were normally distributed and the Mann-Whitney U test was used as the non-parametric test. The time course of responses in different groups of rats were examined for statistical significance by using analysis of variance (ANOVA). $p < 0.05$ was taken to indicate a significant difference. Statistical analyses were performed using either Microsoft Excel version 5.0 or Minitab version 9.

3. MOUSE MAST CELL PROTEASE-1; ISOLATION OF GLYCOFORMS

3.1 Introduction

MMCP-1 isolated from the intestinal mucosa of mice infected with the parasite *Trichinella spiralis*, shares 74% amino acid sequence homology with RMCP II and also has a very similar tissue distribution and biochemical properties (Newlands *et al* 1987; Miller *et al* 1988; Le Trong *et al* 1989; Huntley *et al* 1990a). Murine bone marrow-derived mast cells (BMMC), often regarded as in vitro analogues of MMC (Sredni *et al* 1983), contain four mast cell proteases in the molecular mass range 28-32 kDa including MMCP-1 as determined by Western blotting and ELISA (Newlands *et al* 1991). Four esterases of similar or identical molecular weight which bind the specific serine protease inhibitor [^{3}H]-diisopropylfluorophosphate ([^{3}H]-DFP) have also been found in BMMC (DuBuske *et al* 1984). Five proteases have been identified in CTMC and/or virus-immortalised mast cell lines on the basis of N-terminal amino acid sequences and molecular weights and designated MMCP-2 to MMCP-6 (Serafin *et al* 1990; Reynolds *et al* 1990). All but one of these proteases, MMCP-3, have been cloned and sequenced. Transcripts for MMCP-1 are expressed only in MMC and MMCP-2 is expressed in intestinal mucosa and Kirsten sarcoma virus-immortalised mast cells (KiSV-MC) (Serafin *et al* 1991). MMCP-4 is transcribed in CTMC and KiSV-MC (Serafin *et al* 1991) and MMCP-5 in CTMC and KiSV-MC (Huang *et al* 1991; McNeil *et al* 1991). MMCP-6, identified as a tryptase by its predicted amino acid sequence, appears to be exclusive to CTMC and KiSV-MC (Reynolds *et al*

1991). A second tryptase has also been identified from a transiently expressed cDNA in BMMC and designated MMCP-7 (McNeil *et al* 1992)

The original isolation of MMCP-1, from a homogenate of *T. spiralis*-infected mouse small intestine (Newlands *et al* 1987), showed that the enzyme activity, which hydrolysed a chymotrypsin substrate, was distributed over several peaks of the final Mono S cation exchange chromatogram, in addition to that originally identified as MMCP-1. This suggested that one or more of the other murine mast cell proteases could also be present in the intestinal tissue extract. The proteases present in homogenates from nematode-infected intestine were therefore further analysed to establish their identity. In this chapter the isolation by cation exchange chromatography and biochemical and immunological characterisation of five MMCP-1-like proteases are described and identified as variant forms of MMCP-1 which differ only in their glycosylation. The isolation and partial characterisation of a serosal mast cell protease identified as MMCP-4 are also described.

3.2 Results

3.2.1 Enzyme isolation

3.2.1.1 Enteric proteases.

Cation exchange chromatography (2.1.2) of small intestinal homogenate in 20 mM Tris/HCl buffer pH 7.5 on CM-Sepharose, when eluted with a linear 0-1.0 M NaCl gradient, gave one major peak, which contained all the detectable enzyme activity against the synthetic substrate CBZ-L-Tyr-4NPE. After buffer exchange into 50 mM morpholinoethane sulphonic acid (MES) pH 6.0 (2.1.2) the enzyme activity

was resolved into 5 distinct peaks by FPLC cation exchange chromatography on Mono-S (Figure 1). Each peak containing enzyme activity was collected separately and, after five-fold dilution in 50 mM MES, re-chromatographed on Mono-S where each eluted at a different molarity (88, 92, 103, 109 and 115 mM NaCl for peaks 1 to 5 respectively). These fractions were labelled MMCP-1A to 1E, in order of elution, for identification.

Analysis of the peaks by SDS-PAGE under reducing conditions (2.1.5; Figure 2a) showed MMCP-1A to contain 2 polypeptides of 32 and 33 kDa of different staining intensities, which were not resolved by the techniques used, MMCP-1B and 1C each contained single proteins of 31 kDa, MMCP-1D and 1E each contained a single band of 30 kDa.

3.2.1.2 Serosal mast cell protease;

Size exclusion chromatography of a serosal mast cell granule extract in 20 mM tris/HCl, pH 7.5, 1.0 M NaCl, on sephacryl S-200 resolved two major peaks the second of which contained enzyme activity which hydrolysed CBZ-L-TYR-4NPE. When this material, diluted with 20 mM tris/HCl, pH 7.5 to 0.5 M NaCl, was applied to Mono-S cation exchanger a single peak of bound material was eluted at 0.77 M NaCl. The protein resolved by cation exchange chromatography hydrolysed CBZ-L-TYR-4NPE and contained a single 28 kDa polypeptide by SDS-PAGE (Figure 2a).

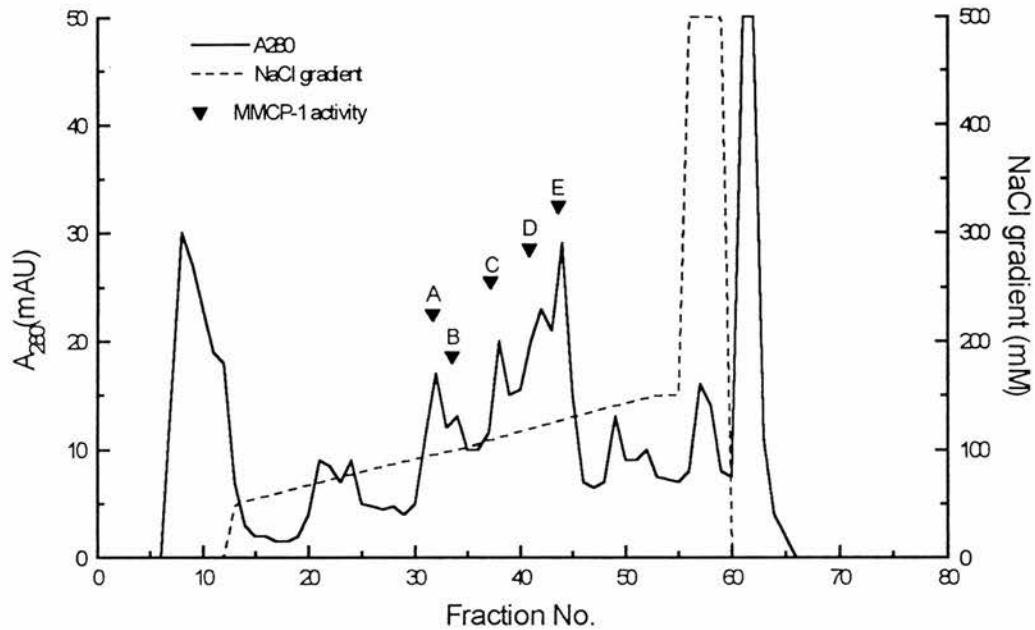


Figure 1. Typical FPLC chromatogram showing the resolution of five peaks of chymotrypsin-like enzyme activity from homogenate of small intestine by Mono-S cation exchange.

Approximately 4.8 mg of protein, eluted from a CM-sepharose cation exchange column, was loaded onto a Mono-S cation exchange column which was equilibrated with 50 mM MES pH 6.0 and eluted with a segmented gradient over 50 to 150 mM NaCl. The peaks of enzyme activity, indicated by arrow heads, were detected by using the chromogenic substrate CBZ-L-Tyr-4NPE. The peaks were designated MMCP-1 A (arrowhead on left) to MMCP-1 E (arrowhead on right) for identification purposes. The peaks were collected separately, re applied to Mono-S, and eluted in highly purified form. The peaks, in order of elution contained the following protein concentrations; A, 2.5 ml @ 77 $\mu g.ml^{-1}$; B, 1.5 ml @ 112 $\mu g.ml^{-1}$; C, 3.0 ml @ 106 $\mu g.ml^{-1}$; D, 2.5 ml @ 146 $\mu g.ml^{-1}$; E, 3.5 ml @ 147 $\mu g.ml^{-1}$; (full details are given in section 2.1.2 of Materials and Methods).

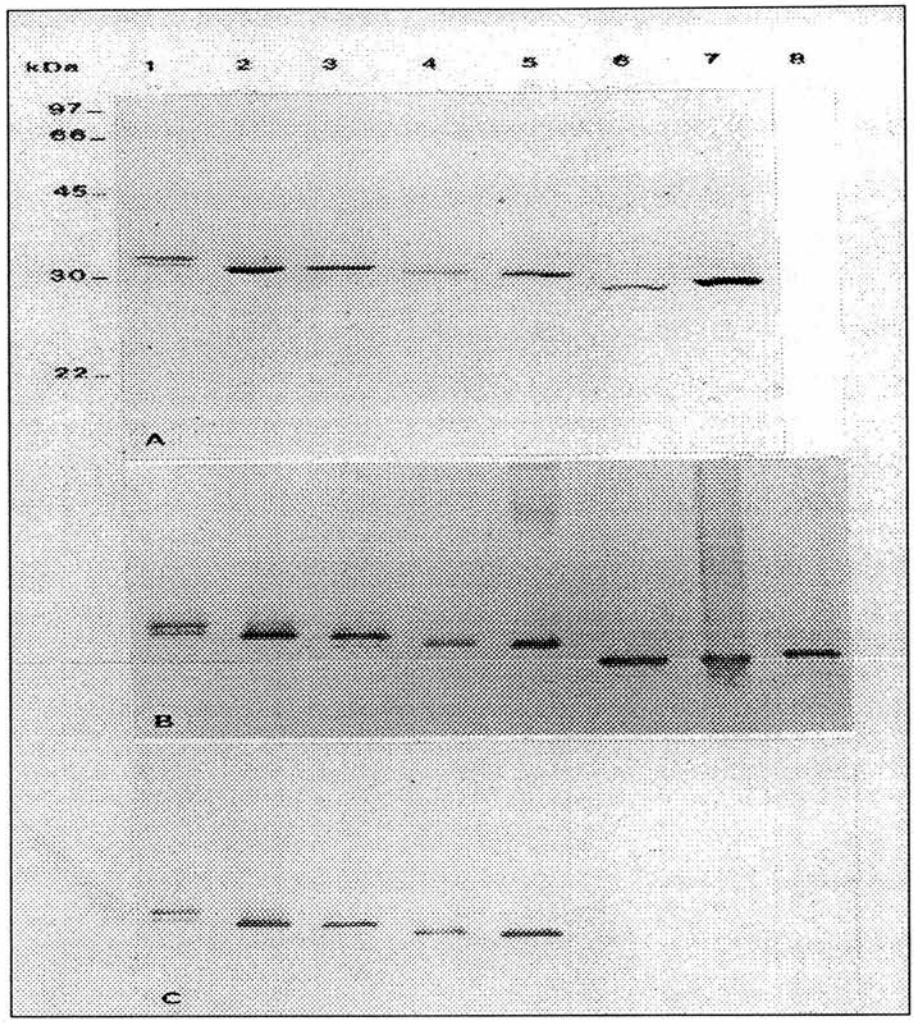


Figure 2. Characterisation of MMCP-1 peaks from Mono S cation exchange by SDS-PAGE and Western blotting.
The proteins were run on 15% discontinuous SDS-PAGE gels. 2A; Peaks 1A-E from Mono S are loaded in lanes 1-5 respectively. Mouse serosal mast cell protease is in lane 6 and rat mast cell protease I in lane 7. The proteins were detected by silver staining. 2B and 2C; Western blot analysis showing MMCP peaks 1A-E from Mono S in lanes 1-5 respectively, mouse serosal mast cell protease in lane 6, 10^4 purified mouse serosal mast cells in lane 7 and RMCP I in lane 8 probed with B, rabbit anti-MMCP-1 or C, sheep anti-MMCP-1 absorbed to remove anti-RMCP I activity. Rabbit and sheep immunoglobulins were detected with sheep anti-rabbit HRPO or pig anti-sheep HRPO conjugates respectively and HRPO activity was revealed by using 3,3` diaminobenzidine as substrate. Isolated enzymes were loaded at the rate of 40-60 ng per lane. Details of the protocols are given in sections 2.1.5 and 2.1.6 of Materials and Methods.

3.2.2 Biochemical characterisation

The MMCP-1 polypeptides in the 30 to 33 kDa range all bound the group-specific inhibitor [^{3}H]-DFP (Figure 3) indicating the presence of serine esterase activity (2.1.11). The autoradiograph of the labelled proteins showed the same pattern as the silver-stained SDS-PAGE gel (Figure 2a) except that MMCP-1D had an additional polypeptide of 27 kDa labelled with [^{3}H]-DFP which was not detectable on the stained gel (Figure 3). This additional polypeptide may represent a degradation product generated during incubation with [^{3}H]-DFP. The five MMCP-1-like enzymes and MSMCP were screened against a range of low molecular weight, synthetic, protease substrates (2.1.15.1) and were found to exhibit chymotrypsin-like esterase activity, as reported previously for MMCP-1 (Newlands *et al* 1987), the results of which are summarised in Table 2. MSMCP also had chymotrypsin-like substrate specificities (Table 2). All five intestinal MMCPs and MSMCP were completely inhibited by PMSF and 3,4 DCI, both inhibitors of serine proteases, but not by 1,10 phenanthroline, E64 or pepstatin, which inhibit metallo-, thiol- and aspartyl proteases respectively (Table 3; 2.1.15.1). The MMCP-1-like proteases were also inhibited by human α_1-PI (2.1.15.2) while MSMCP, like RMCP I in the rat (Pirie-Shepherd *et al* 1991), was not.

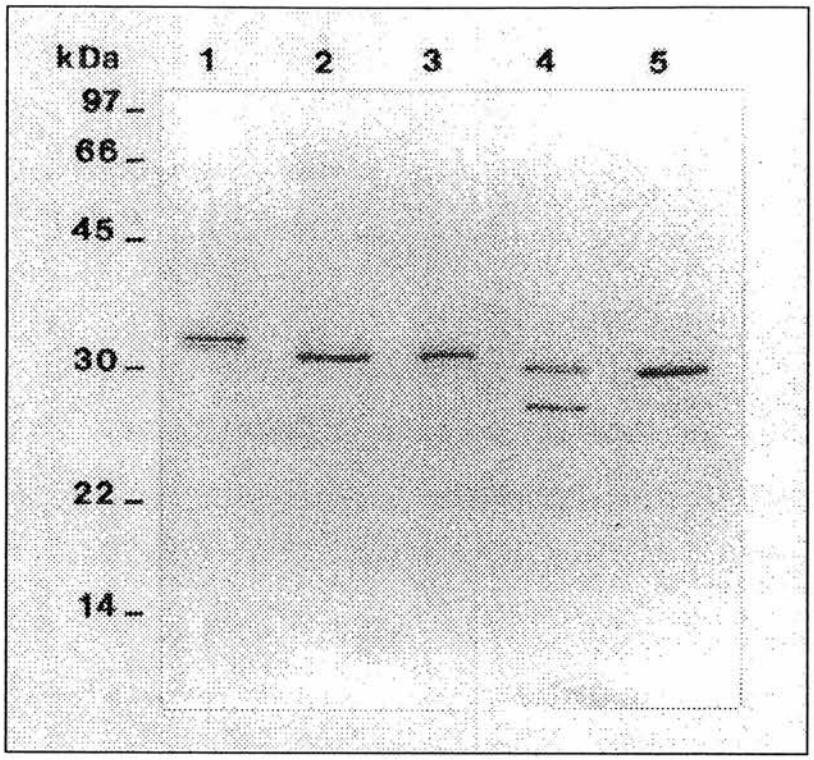


Figure 3. Autoradiograph of MMCP-1 peaks 1A-E labelled with the specific serine esterase inhibitor [^{3}H]-DFP.
Labelled proteases were loaded at 2 μg/lane on a 12% SDS-PAGE gel and run under reducing conditions.

Table 2. Substrate specificities of multiple forms of MMCP-1 and of MSMCP.

		Activity (nkat/mg)					
	Substrate	1A	1B	1C	1D	1E	MSMCP
Chymotryptic (esterase)	CBZ -L-Tyr-4NPE	63	42	31	33	21	259
	CBZ -L-Phe-4NPE	14	10	6	5	4	9
	CBZ -L-Trp-4NPE	0.3	0.9	NA	0.2	NA	NA
Elastinolytic (esterase)	CBZ -L-Ala-4NPE	15	6	4	9	4	41
	CBZ -D-Ala-4NPE	8	2	2	5	2	22
Tryptic (esterase)	CBZ -L-Lys-4NPE	NA	NA	NA	NA	NA	NA
Chymotryptic (amide)	Bz-L-Tyr-4NA	1	NA	NA	NA	NA	2
	Succ-Phe-4NA	0.8	0.2	NA	NA	0.3	5
Tryptic (amide)	CBZ-L-Arg-4NA	2	NA	NA	0.4	NA	8
	Bz-DL-Arg-4NA	1	0.3	0.001	NA	NA	NA

Enzyme activity is expressed in nkat/mg of protein; 1 nkat is that activity which hydrolyses 1 nMol of substrate in 1 second. NA = no activity (2.1.12).

Table 3. Inhibition characteristics of mast cell proteases MMCP-1A-E and MSMCP.

Inhibitor	1A	1B	1C	1D	1E	MSMCP
PMSF	100	100	100	100	100	100
3,4 DCI	100	100	100	100	100	100
E64	5	18	5	9	0	0
1,10 Phe	8	5	11	5	0	0
Pepstatin	13	0	0	0	7	0

Percentage inhibition after 60 min incubation with inhibitor. n = 3 for each preparation. PMSF, phenyl-methyl-sulphonyl-fluoride (serine protease inhibitor); 3,4 DCI, 3,4-Dichloro-isocoumarin (serine protease inhibitor); E64, N-[N-(L-3-Trans-carboxyoxirane-2 carbonyl)-L-leucyl] -agmatine (thiol protease inhibitor); 1,10-Phe, 1,10-phenanthroline (metallo-protease inhibitor); pepstatin (aspartic protease inhibitor). The relative concentrations of inhibitors and proteases is described in Materials and Methods section 2.1.15.1.

3.2.3 Immunological analysis

The isolated MMCPs were all detected with the rabbit antiserum raised against MMCP- (Newlands *et al.*, 1987; Figure 2b; Materials and Methods, 2.1.10) on Western blot as was the 28 kDa MSMCP (Figure 2b). When a similar blot was probed with sheep anti-MMCP-1, cross-absorbed against RMCP I (2.1.10), only the highly soluble antigens isolated from small intestine were identified (Figure 2c).

The five MMCP-1-like enzymes shared complete lines of identity in gel diffusion against sheep anti-MMCP-1 whereas no precipitation occurred with MSMCP (Miller *et al* 1990). When sheep anti- MMCP-1 was substituted with sheep

anti- RMCP I a precipitin line was uniquely present against MSMCP and there was no reaction against MMCP-1 (Miller *et al* 1990).

The five intestinal MMCPs (0.5 to 12 ng/ml) were detected by ELISA (2.1.8), using sheep anti-MMCP-1 as capture antibody, whereas MSMCP, RMCP I and RMCP II were not detected at 270 ng, 2.5 mg and 5.0 mg/ml respectively. After establishing the specificity of the ELISA, homogenates of small intestine from *N. brasiliensis*-infected mice were assayed (2.1.8) and MMCP-1 concentrations were found to be increased 14 fold when compared with uninfected control mice (Table 4). In contrast, homogenates of tongue from control mice had no detectable MMCP-1 despite being rich in mast cells (Table 4; Figure 4). Small quantities of MMCP-1 were detected in tongue of infected mice (Table 4) and, as described previously this is probably derived from blood, where levels of MMCP-1 are raised in nematode infection (Huntley *et al.*, 1990a).

Immunohistochemical studies using the directly labelled sheep anti-MMCP-1-FITC confirmed the ELISA results in that there were increased numbers of mast cells in the intestinal mucosa of parasitised mice which fluoresced after incubation with sheep anti-MMCP-1-FITC conjugate when compared with uninfected controls (Figure 4, Table 4). There was no cellular immunofluorescence in tongue of either control or parasitised mice despite the presence of abundant mast cells which stained with toluidine blue (Figure 4).

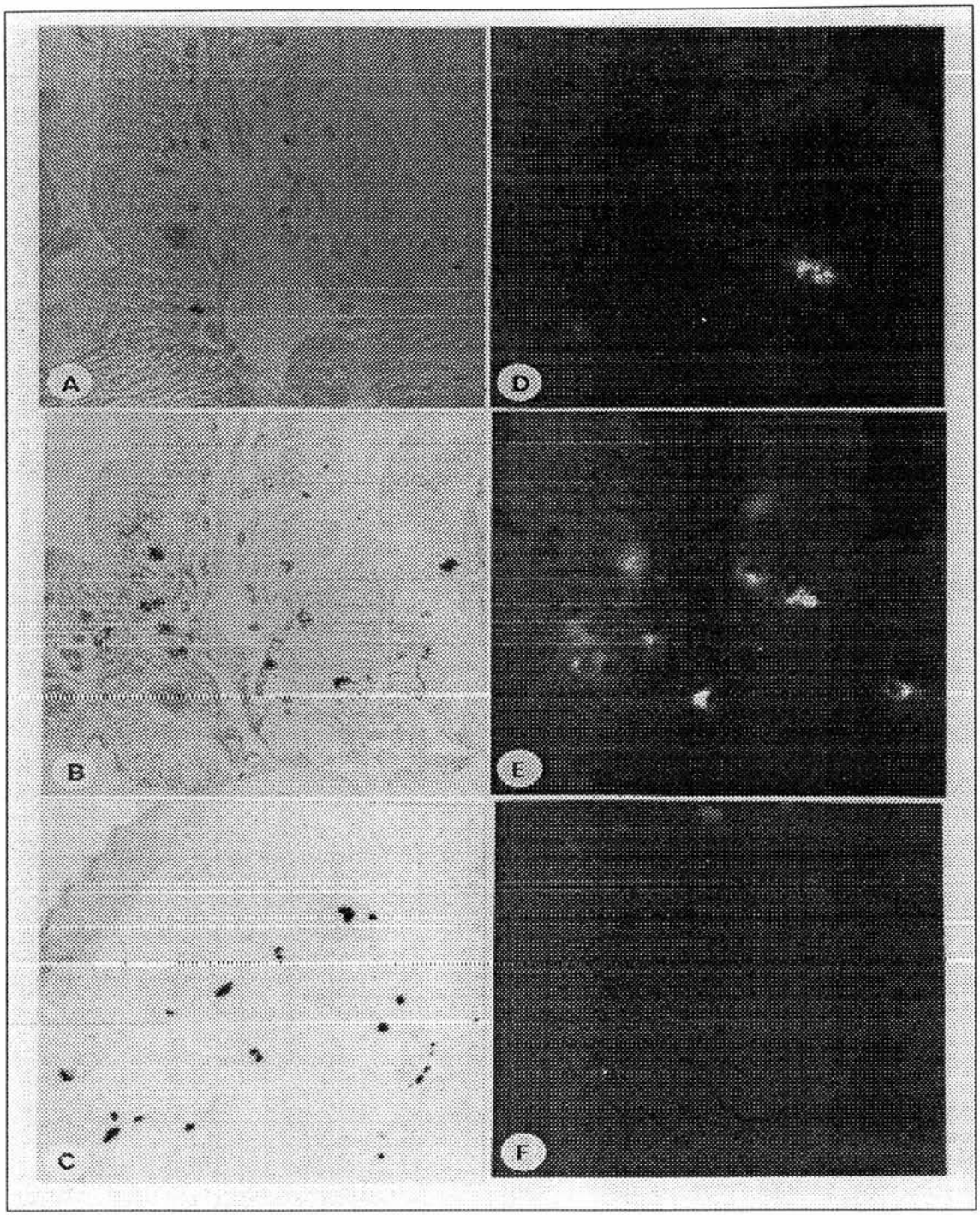


Figure 4. Photomicrographs showing normal mouse small intestine, *T. spiralis*-infected small intestine and normal mouse tongue stained with toluidine blue pH 0.5 (A-C respectively) or sheep anti-MMCP-1-FITC (D-F respectively).

Table 4. MMCP concentrations and mast cell numbers in *N. brasiliensis*-infected and normal mice.

	Control		Infected	
	Jejunum	Tongue	Jejunum	Tongue
MMCP (μg/g)•	13 ± 3.6	0	183 ± 23.7	0.5 ± 0.12
Mast cell/vcu (tol. blue)	1 ± 0.2	A*	7 ± 0.7	A*
Mast cell/vcu (fluorescence)	1 ± 0.3	0	6 ± 0.3	0

Jejunal mast cell counts are expressed as cells per villus/crypt unit (vcu). A* = mast cells were abundant but were not counted. • μg/g wet weight of tissue.

3.2.3.1 N-terminal sequencing

To determine whether the five intestinal mast cell proteases were separate gene products or differed because of post-translational processing, the amino-terminal amino acid sequence was determined for each protease, the results of which are summarised in Figure 5. The first 15 to 29 amino-terminal residues of the intestinal mast cell proteases proved to be identical in each case and were in agreement with the previously published sequence for mouse intestinal mast cell protease (Le Trong *et al.*, 1989). The amino acid sequence determined for MSMCP showed complete identity over the first 20 residues with that published for MMCP-4 (Reynolds *et al.*, 1990).

3.2.3.2 Deglycosylation

To further investigate whether the differences between MMCP-1 A-E were post-translational and a consequence of differential glycosylation, samples of each enzyme preparation were incubated with endo-α-acetyl galactosaminidase to remove o-linked carbohydrate or with peptide-N-glycosidase F (PNGase F) to remove N-linked carbohydrates. The protocols are described in Materials and Methods, section 2.1.7. Briefly, 2 μg of protein were denatured by heating in a boiling water bath, cooled and incubated with 0.2 units of the appropriate glycosidase at 37°C overnight. No change in the apparent molecular weight of any of the MMCPs, as determined by SDS-PAGE was detected even after prolonged treatment with o-glycosidase. Following treatment of the five MMCPs with PNGase F, however, there was degradation of each MMCP resulting in the appearance of a new polypeptide with an apparent molecular weight of 28 kDa (Figure 6). This 28 kDa polypeptide remained strongly antigenic on Western blot when probed with sheep anti-MMCP 1 (not shown).

Figure 5. N-terminal amino acid sequences of variant glycoforms of MMCP-1 (A-E) and MSMCP (MMCP-4).

	1				5					10					15					20					25				29
MMCP-1A	I	I	G	G	V	E	A	R	P	H	S	R	P	Y	M	A	H	L	K	I	I	T	D	R	G	S	E	D	R
MMCP-1B	I	I	G	G	V	E	A	R	P	H	S	R	P	Y	M														
MMCP-1C	I	I	G	G	V	E	A	R	P	H	S	R	P	Y	M	A	H	L	K	I	I	T	D	R	G	S	E	D	R
MMCP-1D	I	I	G	G	V	E	A	R	P	H	S	R	P	Y	M	A	H	L	K	I	I	T	D	R	G				
MMCP-1E	I	I	G	G	V	E	A	R	P	H	S	R	P	Y	M	A	H	L	K	I	I	T	D	R	G				
MSMCP	I	I	G	G	V	E	S	R	P	H	S	R	P	Y	M	A	H	L	E	I									

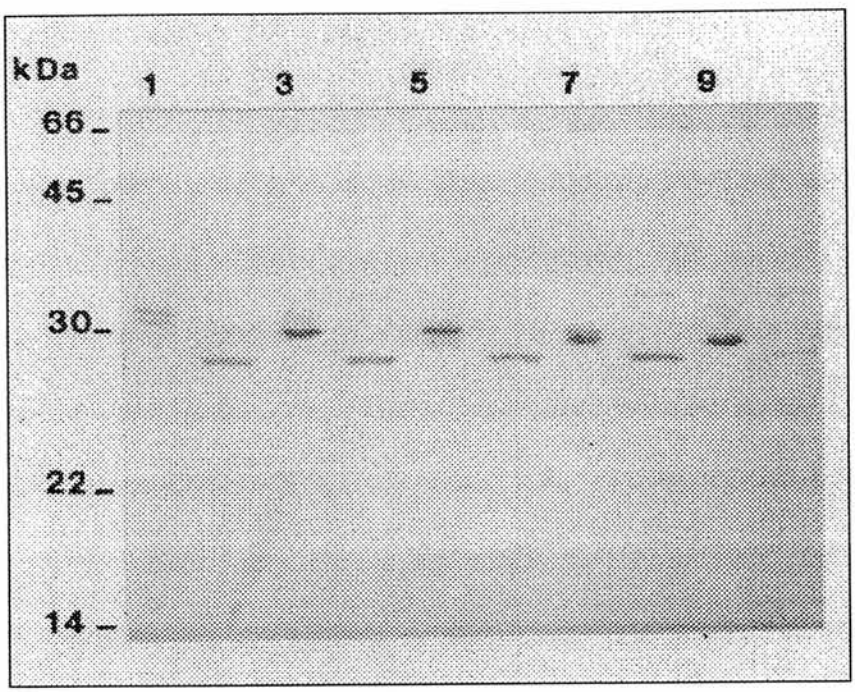


Figure 6. Treatment of MMCP-1 glycoforms with PNGase F to remove N-linked carbohydrate.
Silver-stained 15% SDS-PAGE gel showing MMCP 1A to 1E before (lanes 1, 3, 5, 7 and 9 respectively) and after (lanes 2, 4, 6, 8, and 10 respectively) removal of N-linked carbohydrate with PNGase F. The enzymes were loaded at 20 ng/lane.

3.3 Discussion

The five proteases isolated from *T. spiralis*-infected mouse small intestine can be identified as MMCP-1 by several criteria. Like MMCP-1, they are highly soluble, chymotrypsin-like proteases active at neutral pH. They are closely related antigenically both on Western blot and by Ouchterlony double diffusion and are readily distinguished from MSMCP on the basis of their antigenicity. Finally, they share complete amino acid sequence identity over the first 15-29 N-terminal residues

(Le Trong *et al* 1989) which further distinguishes the MMCP-1 group of proteases from MMCPs-2-6 (Serafin *et al* 1990; Reynolds *et al* 1990) all of which differ from MMCP-1 within the first 10 N-terminal residues.

The differences in electrophoretic mobility between these variant forms of MMCP-1 are probably a result of variable glycosylation since removal of N-linked carbohydrate moieties produced a new polypeptide of approximately 28 kDa from each of MMCP-1 A-E which was antigenically similar to native MMCP-1. Analysis of the carbohydrate content of each enzyme preparation might prove helpful in further distinguishing between the different glycoforms of MMCP-1. It may be that each is produced by mast cells in different regions of the intestine or, more likely, by mast cells at varying stages of maturation since intestinal nematodiasis induces massive mucosal mastocytosis with extensive recruitment and differentiation of intestinal mast cells (Miller *et al* 1988).

Variable glycosylation occurs in other serine proteases notably in the sub-units of human mast cell tryptase (Cromlish, Seidah, Marcinkiewicz, Hamelin, Johnson & Chretien, 1987) and tissue plasminogen activator (t-PA) (Wittwer, Howard, Carr, Harakas, Feder, Parekh, Rudd, Dwek & Rademacher, 1989). Human tissue plasminogen activator is synthesised in two forms, I and II, which share N-terminal amino acid sequences but have disparate carbohydrate side groups, the composition of which appears to be dependent on the cell line from which the tPAs are derived (Wittwer *et al.*, 1989). In the presence of a fibrinogen fragment stimulator, t-PA II will catabolise a synthetic substrate up to five times faster than t-PA I (Wittwer *et al.*, 1989) whilst type I is more resistant to cleavage by plasmin (Wittwer & Howard

1990). Human mast cell tryptase has also been described as having variable Mr of between 28 and 37 kDa and an isoelectric point of between 5.0 and 6.5, properties which depend on glycosylation (Benyon, Enciso & Befus, 1993).

MSMCP, isolated from peritoneal CTMC, is the first murine CTMC protease to be isolated in its native form and characterised biochemically. MSMCP is a neutral 28 kDa serine protease with chymotrypsin-like substrate specificities, properties it has in common with MMCP-1. It differs from MMCP-1 in that it is highly insoluble and, on cation exchange chromatography, behaves like RMCP I which has a net charge of +18 (Le Trong *et al.*, 1987). In contrast, MMCP-1 has similar chromatographic properties to RMCP II and has a net charge of +3 at neutral pH (Le Trong *et al.*, 1989). N-terminal amino acid sequence analysis of MSMCP shows complete identity over the first 20 residues with the CTMC protease, MMCP-4 (Reynolds *et al.*, 1990; Serafin *et al.*, 1991), differing at residues 7 and 19 from MMCP 1 A, C, D, and E (MMCP-1B was not sequenced beyond residue 15). Based on this evidence we can identify MSMCP as MMCP-4.

Immunological techniques have proved to be particularly useful in the analysis of protease distribution and function. For example, Woodbury and colleagues (1978) showed that RMCP I and II were readily distinguished by gel diffusion although they share 74% amino acid sequence homology. However it was not possible to determine the cellular distribution of RMCP I and II without first preparing monospecific polyclonal antibodies by cross-absorption (Gibson & Miller, 1986) or by raising a monoclonal antibody against RMCP II (Huntley *et al.*, 1990b). The distinction between the MMCP-1 family and MMCP-4 was, however, readily achieved with

polyclonal sheep anti-MMCP-1 cross-absorbed against RMCP I and affinity purified on MMCP-1-Sepharose 4B. This was true by gel diffusion, Western blotting, ELISA and immunohistochemistry. It was thus possible, using this antibody for immunohistochemistry, to confirm ELISA results (Huntley *et al.*, 1990a) showing that the MMCP-1 family of proteases is not present in CTMC. As yet no antibodies are available which allow distinction between MMCPs 1A-1E so it is not clear whether the different glycoforms are present in distinct regions of the small intestine or are being produced by MMC of varying maturity.

Using cDNA probes and Northern blotting, Serafin and colleagues (1991) detected expression of MMCP-4 in the intestines of parasitised mice. This may be in agreement with our finding of an RMCP I-like antigen expressed in mast cells in the gastrointestinal mucosae of *T. spiralis*-infected mice (Miller *et al* 1988) although, in the rat, RMCP I is not present in intestinal mucosal mast cells (Huntley *et al.*, 1990b). Serafin and colleagues (1991) were, however, unable to detect MMCP-4 transcripts in bone marrow-derived cultured mast cells by Northern blotting, although transcription of MMCP-4 has recently been reported in BMMC grown in medium containing the *c-kit* ligand growth factor (Gurish, Ghildyal, McNeil, Austen, Gillis & Stevens, 1992). An RMCP I-like antigen in murine BMMC, detected with anti-RMCP I by immunofluorescence and by immunoblotting of cell lysates, has a very similar or identical electrophoretic mobility to the mouse CTMC protease now identified as MMCP-4 (Newlands *et al.*, 1991). It is therefore probable that MMCP-4 is present in BMMC grown in T cell conditioned medium and the failure to detect it by Northern blotting may simply be due to the low levels of transcription. For example, murine

BMMC contain <120 ng MMCP-1/10^6 cells (Newlands *et al.*, 1991) compared with up to 34 μg RMCP II/10^6 rat BMMC (Haig *et al.*, 1988). These results again emphasise the potential value of highly specific antibodies in the analysis of protease distribution.

In summary, the five proteases isolated from mouse small intestine are identified as variant glycoforms of MMCP-1 and the novel CTMC protease is identified as MMCP-4. The biological significance of so many forms of MMCP-1 will only become clear when their native substrates and specific inhibitors are identified and their interactions characterised.

4. MOUSE MAST CELL PROTEASE-1; SUBSTRATE AND INHIBITOR KINETICS

4.1 Introduction

The identification of several different glycoforms of MMCP-1 (chapter 3) that differ only in their carbohydrate moieties raises interesting questions about the possible effects of variable glycosylation on the functional properties of these enzymes. Many aspects of serine protease function can be modified through their carbohydrate side chains including secretion, affinity for co-factors, substrate affinity, activation rate, susceptibility to inhibition and clearance time from the circulation.

The presence of N-linked glycosylation at one specific site of several potential glycosylation sites is essential for the efficient secretion of a recombinant human anti-thrombotic protease, protein C from a mammalian cell line (Grinnell, Walls & Gerlitz, 1991), whilst the affinity of antithrombin for its co-factor heparin, which enhances enzymic activity, is increased or decreased depending on the degree of glycosylation of the enzyme (Bjork, Ylinenjarvi, Olson, Hermentin, Conradt & Zettlmeissl, 1992). The rate at which plasminogen is converted to plasmin, on a fibrin substrate, by tissue-type plasminogen activator (t-PA) is significantly increased with decreased sialation of the carbohydrate side-chains (Pirie-Shepherd, Jett, Andon & Pizzo, 1995). Interestingly, t-PA is itself secreted in two glycoforms (discussed in chapter 3) which will hydrolyse a synthetic substrate at significantly different rates (Wittwer *et al* 1989) whilst one form is more resistant to inactivation (Wittwer *et al* 1989). It is also suggested that a non-glycosylated, recombinant plasminogen has a decreased circulation time *in vivo* compared with the glycosylated form of the enzyme

(Gonzalez-Gronow, Grenett, Fuller & Pizzo, 1990). Taken together this evidence shows that glycosylation influences most aspects of serine protease function and it is therefore important to determine the effects of variable glycosylation on MMCP-1 function.

In order to establish whether these variations in glycosylation modified MMCP-1 activities a number of kinetic parameters were examined; the Michaelis constant (K_m) which indicates the affinity of the enzyme/substrate (ES) complex, the catalytic constant or turnover number (k_{cat}), which represents the rate of substrate hydrolysis at saturating substrate concentrations and the k_{cat}/K_m ratio, a measure of the overall catalytic efficiency (Voet & Voet 1991).

A major factor in regulating the function of proteolytic enzymes is the presence of effective inhibitors which inactivate the enzyme once it has fulfilled its function. Rodent mast cell chymases are inhibited by the α1-proteinase inhibitor (α_1-PI) family of serine proteinase inhibitors (serpins) present in blood (Irvine, Newlands, Huntley & Miller, 1990; Pirie-Shepherd *et al* 1991) and therefore the second order association rate constant (k_{ass}) and dissociation constant for the enzyme/inhibitor complex (K_i), which represent the rate of enzyme inhibitor complex formation and the stability of that complex respectively, for the reaction between the MMCP-1 proteases and α_1-PI were determined. Protein inhibitors of the α-$_1$ PI class function as inhibitors as a consequence of binding of the substrate-binding, active site region of the protease to a corresponding, substrate-like, reactive-site region on the surface of the inhibitor (Travis & Salvesen 1983). Near the centre of the reactive-site there is an amino acid residue, recognised by the target protease's substrate-binding active-site, termed the

P1 residue (Travis & Salvesen 1983). The protease cleaves the peptide bond between the P1 residue and the residue immediately adjacent to it on the carboxy terminal side, termed the P'1 residue. Hydrolysis of this reactive site peptide bond does not proceed to completion but rather an equilibrium is formed between the intact and cleaved forms of the inhibitor which forms a stable complex with the protease (Travis & Salvesen 1983). To accurately calculate these parameters it was important initially to establish the precise concentration of each of the enzyme preparations. Since a proportion of the enzyme present in solution could well be inactive, using an estimate of the protein content to determine enzyme concentration was inappropriate and the concentration was determined by active site titration with a suitable substrate.

4.2 Results

4.2.1 Active site titration

Concentrations of functional protease were determined by spectrofluorimetric titration of the active-sites of the MMCP-1-like proteases against the substrate 4-methylumbelliferyl-p-(NNN trimethylammonium) cinnamate (MUTMAC; Jameson *et al* 1973; 2.1.14). Chymases form stable, inactive, acyl compounds with this substrate and liberate 4-methylumbelliferone (7-hydroxy-4-methylcoumarin) with a negligible rate of de-acylation (Jameson *et al* 1973). A standard curve was prepared from 7-hydroxy-4-methylcoumarin and the relationship between concentration and fluorescence intensity was linear over the range 2.5 to 40 nM (Figure 7).

Unknown samples were assayed, in quadruplicate, and concentrations of proteases were calculated from linear regression Equation 3.

Equation 3 Linear regression of fluorescence intensity on enzyme concentration.
$y = -9.8 + 22.3x$.

Where y is fluorescence intensity and x is the protease concentration.

The concentrations of protease, as determined by spectrofluorometric titration were similar to those estimated from the total protein assay and are summarised in Table 5. This method of active site titration compares very favourably with colourimetric methods in terms of sensitivity e.g. the spectrofluorimetric titration of MMCP-1A reproducibly measured 14.6 pM of enzyme in the assay mixture.

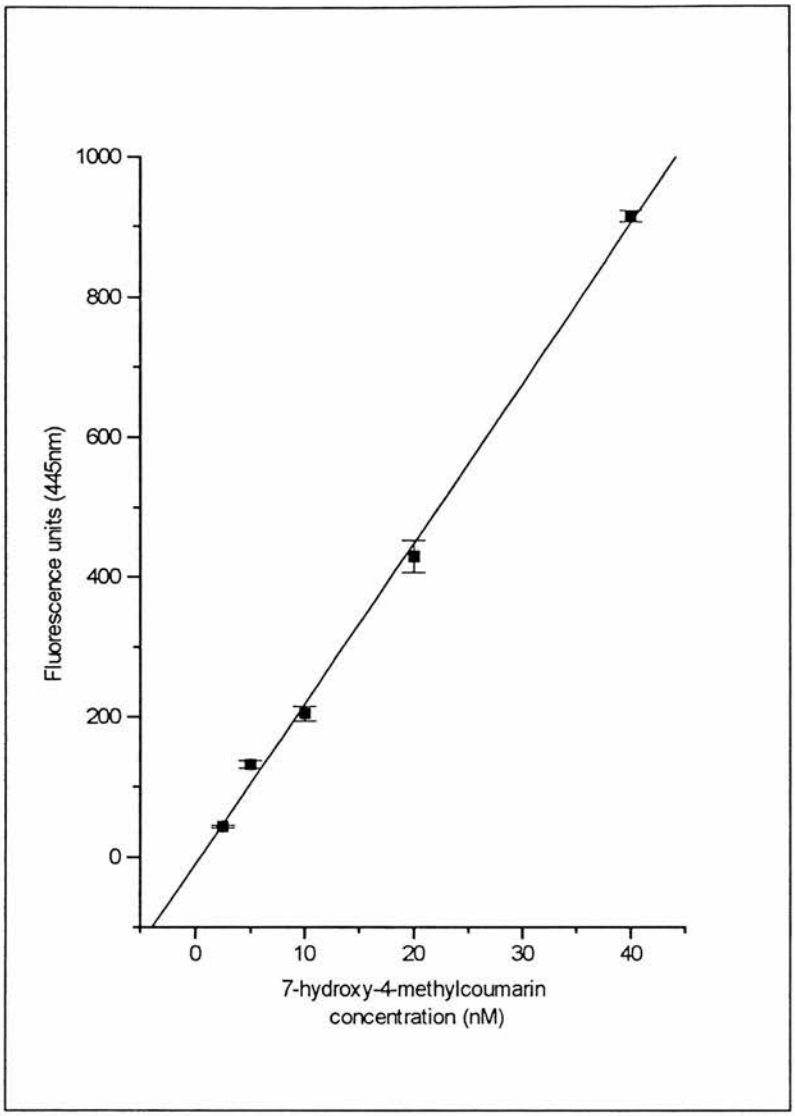


Figure 7 Standard curve prepared from 7-hydroxy-4-methylcoumarin for active site titration of MMCP-1 proteases.

The standard curve was prepared as described in Materials and Methods section 2.1.14 ($R = 0.99$, $p < 0.0001$) and the concentration of unknown samples were calculated from Equation 3.

A colourimetric titration, carried out at pH 3.2 of the same enzyme preparation with CBZ-L-Tyrosine-4-nitrophenyl ester as substrate (Kezdy & Kaizer, 1970) would have an absorbance of < 0.0001 at 340 nm given that the molar extinction of the reaction product is 6360 $M^{-1}.cm^{-1}$. This is at the extreme lower end of the working range of most spectrophotometers and could not be regarded as an accurate or reproducible means of determining the concentration of active sites.

Table 5 MMCP-1 glycoform concentrations by spectrofluorimetric titration of active sites.

Protease	Protease concentration by Spectrofluorimetry (μM)	Protease concentration by Protein assay (μM)
1 A	2.91 ± 0.10	2.37
1 B	3.99 ± 0.03	3.61
1 C	2.36 ± 0.05	3.42
1 D	5.09 ± 0.18	4.87
1 E	4.53 ± 0.04	4.90

The results of the spectrofluorometric titration of the MMCP-1 enzymes are mean ± sem of four replicate tests.

4.2.2 Substrate Kinetics

All kinetic measurements were made using a Beckman DU 600 spectrophotometer. Rates of hydrolysis of 5 concentrations (0.125 to 2 mM) Succ-Ala-Ala-Pro-Phe-4NA were measured by adding 2 μl of substrate solution to 48 μl of enzyme solution in 0.1M tris/HCl pH 7.5. The increase in absorbance was

continuously measured at 410 nm and the spectrophotometer's on-board software used to calculate the Michaelis constant (K_m), and maximum velocity (V_{max}) from the Michaelis-Menten rate equation by non-linear regression, where v is the reaction velocity and s the substrate concentration.

Equation 4 Michaelis Menten rate equation.

$$v = \frac{V_{max} \cdot s}{(K_m + s)}$$

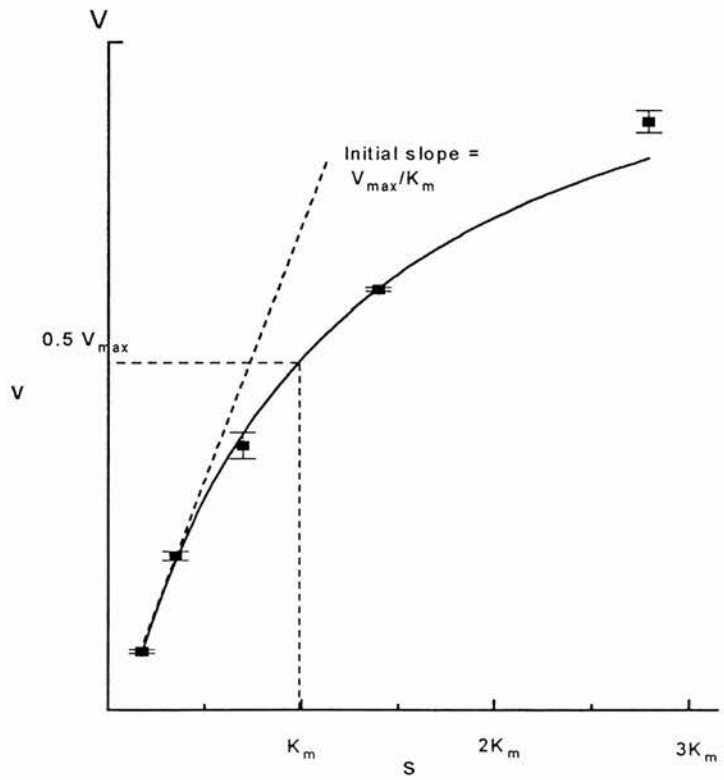


Figure 8 Plot of substrate concentration versus reaction velocity used to calculate K_m and V_{max} by non-linear regression.

This plot is derived from the Michaelis-Menten equation as described in Equation 4.

From these parameters it is possible to calculate the catalytic constant or turnover number (k_{cat}) from

Equation 5 Calculation of the catalytic constant k_{cat}.

$$k_{cat} = \frac{V_{max}}{E^0}$$

where E^0 is the enzyme concentration in the reaction mixture as determined by active site titration.

Using Succ-Ala-Ala-Pro-Phe-4NA as substrate (2.1.13), kinetic constants were calculated for each of the five MMCP-1-like enzymes. There were significant differences in K_m values ($p = 0.025$) with this substrate and highly significant differences in both k_{cat} and k_{cat}/K_m ($p<0.0001$ for each) by one-way analysis of variance. These results are summarised in Table 6. The pH optima lay in the range pH 7.5-7.8 for all of the isolated enzymes.

4.2.3 Inhibitor kinetics

The second order association rate constant (k_{ass}) for the reaction between the MMCP-1-like proteases and human α_1 proteinase inhibitor (α_1-PI; Sigma) was determined by pre-incubating equimolar enzyme and α_1-PI for periods of 15 to 120s before measuring the rate of hydrolysis of the substrate Succ-Ala-Ala-Pro-Phe-4NA (2.1.15.2). The k_{ass} for each reaction was calculated by non-linear regression (Figure 9) as described previously (Pirie-Shepherd *et al* 1991) from the following equation.

Equation 6 Calculation of the association rate constant k_{ass}.

$$E = \frac{E^0}{(1 + E^0 . k_{ass} . t)}$$

where E is the residual active enzyme, E^0 the initial enzyme concentration and t the time in seconds. The k_{ass} values for the reaction between MMCP-1A - 1E and α_1-PI were significantly different by ANOVA ($p = 0.001$) with results in the range of 3.3 - 12.3 x 10^5 $M^{-1}.s^{-1}$ (Table 6).

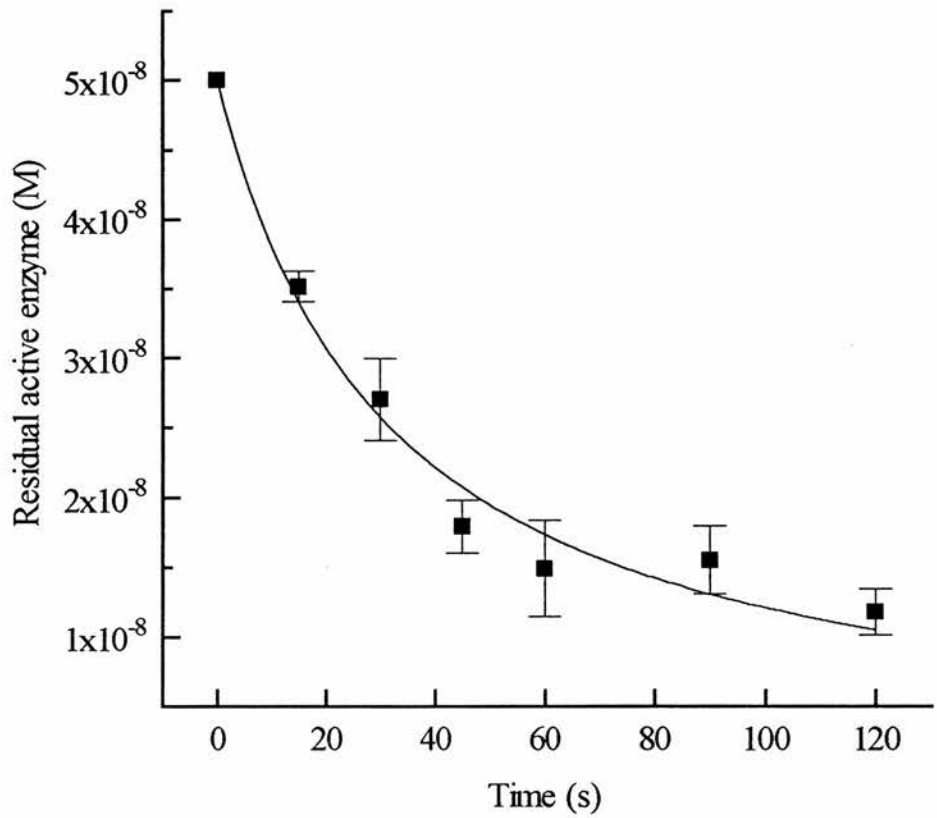


Figure 9 Plot of residual active enzyme versus time used to determine the enzyme/inhibitor association constant, k_{ass} for the reaction between MMCP-1A and α_1-PI by non-linear regression using Equation 6.

The inhibition constant (K_i) for the reaction between each protease and α_1-PI was determined by incubating protease with varying inhibitor concentrations (0.25 to 1.75 molar equivalents) for 35 times $t_{1/2}$, the time to half inhibition for an equimolar reaction, (2.1.15.2), calculated from:-

Equation 7; Time to half inhibition for equimolar enzyme and inhibitor.

$$t_{1/2} = 1 / k_{ass}.E^0$$

where E^0 is the initial enzyme concentration. The apparent K_i (K_i^{app}) was calculated by non-linear regression (Figure 10), as described previously (Pirie-Shepherd *et al* 1991) from Equation 8;

Equation 8 Calculation of K_i by non-linear regression.

$$\frac{E}{E^0} = 1 - \frac{([E^0]+[I^0]+K_i) - \sqrt{([E^0]+[I^0]+K_i)^2 - (4[E^0][I^0])}}{2[E^0]}$$

(Bieth 1974) and the true K_i calculated from Equation 9;

Equation 9 Formula for calculation of the true K_i from the apparent K_i.

$$K_i = \frac{K_i^{app}}{1 + S^0/K_m}$$

where S^0 is the initial substrate concentration.

The MMCP-1-like proteases were all inhibited by human α_1-PI while MSMCP, like RMCP I in the rat, was not (Pirie-Shepherd *et al* 1991). The k_{ass} for the reactions between the MMCP-1-like proteases and α_1-PI showed highly significant differences by one way analysis of variance ($p = 0.001$). Inhibition constants obtained for the interaction of the MMCPs with α_1-PI showed K_is in the 0.8 - 45 pMolar range with the K_i of MMCP-1C being almost 60-fold greater than that of MMCP-1A ($p<0.0001$; Table 6).

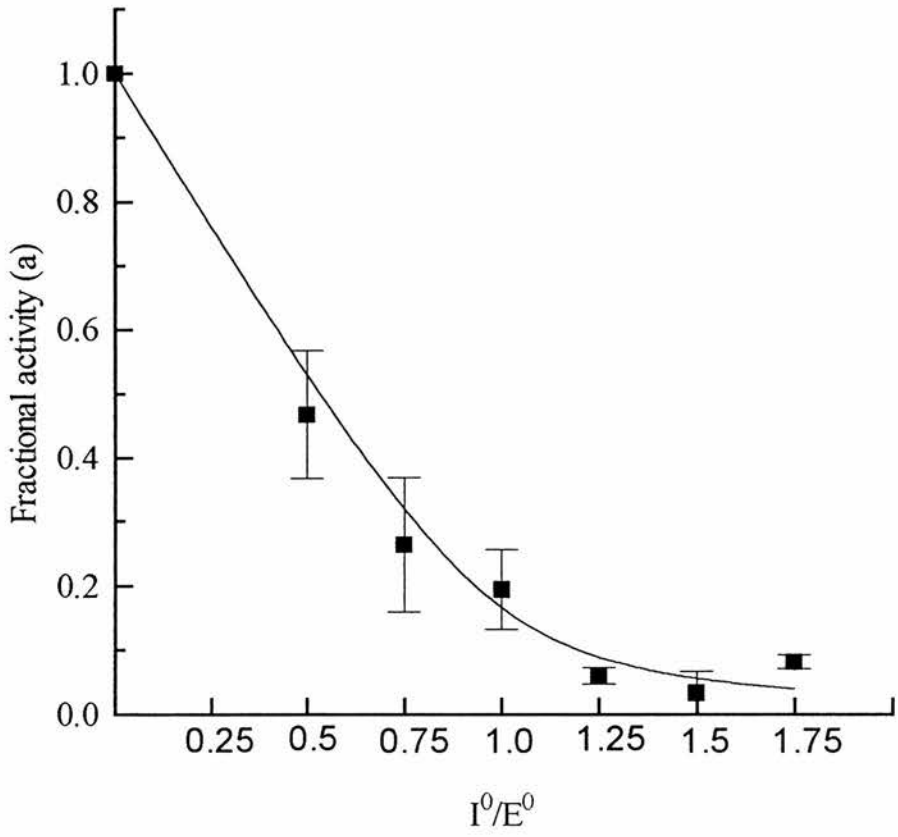


Figure 10 Plot of inhibitor/enzyme ratio versus fractional activity for the reaction between MMCP-1A and α_1-PI used to determine K_i by non-linear regression using Equation 8.

Table 6 Substrate and inhibitor kinetic parameters of the MMCP-1-like proteases.

MMCP	K_m (μM)	k_{cat} (s^{-1})	k_{cat} / K_m ($x10^3 M^{-1}.s^{-1}$)	k_{ass} ($x10^5 M^{-1}.s^{-1}$)	K_i (pM)
1A	922 ± 73.7	2.33 ± 0.10	2.5 ± 0.09	7.67 ± 1.50	0.76 ± 0.18
1B	991 ± 59.9	1.66 ± 0.04	1.7 ± 0.07	12.30 ± 0.35	1.02 ± 0.10
1C	663 ± 49.9	2.84 ± 0.12	4.3 ± 0.22	3.29 ± 0.48	45.40 ± 7.90
1D	936 ± 83.9	2.54 ± 0.12	2.7 ± 0.13	4.07 ± 0.62	2.20 ± 0.34
1E	832 ± 56.9	1.25 ± 0.04	1.5 ± 0.07	11.40 ± 0.22	1.51 ± 0.12
n	4	4	4	3	3
ANOVA	P=0.026	P<0.0001	0<0.0001	P=0.001	P<0.0001

Conditions for substrate kinetics; 0.1M tris/HCl pH 7.5 at 22°C.

4.3 Discussion

The MMCP-1 proteases, designated here MMCP-1A to 1E, have been identified as probably having an identical polypeptide core by several different criteria, and differing only in their carbohydrate side chains (Newlands, Knox, Pirie-Shepherd, & Miller, 1993; chapter 3). It was the aim of the work reported in this chapter to determine whether these differing carbohydrate moieties exert an influence on the *in vitro* functional properties of the enzyme glycoforms in their interactions with synthetic peptide substrate and with a specific protein inhibitor of serine proteases. The kinetic data presented here show that there are significant differences in the K_m values obtained for the MMCP-1 proteases with the low molecular weight, synthetic peptide substrate Succ-Ala-Ala-Pro-Phe-4NA. These were in the range 663 - 991 μM which was similar to the K_m values for RMCP II which were in the range 840 - 910 μM with similar substrates (Powers, Tanaka, Harper, Minematsu, Barker, Lincoln, Crumley, Fraki, Schechter, Lazarus, *et al* 1985). The k_{cat}/K_m ratio for the isolated proteases is in the range 1.5 - 4.3 $x10^3$ $M^{-1}.s^{-1}$ indicating that enzymes are moderately efficient in hydrolysing this low molecular weight, synthetic substrate. This is in contrast with rates in the range 10^8 - 10^9 $M^{-1}.s^{-1}$ achieved by some enzymes such as acetylcholine esterase or carbonic anhydrase when acting on their native substrates, rates limited only by the diffusion rates of the reactants (Stryer, 1981). Since the core polypeptide is probably identical in each enzyme, as determined by the studies in chapter 3, it is likely that the differences in catalytic properties are due to the carbohydrate moieties. This may be because the carbohydrate groups which comprise 14-22% of the total native molecule, based on the predicted Mr of the core

polypeptide (Huang *et al,* 1991) and the estimated Mr of the individual glycoforms (3.2.2) may alter the folding of the polypeptide chain and thus changing the conformation of the active site cleft or, more likely by direct steric hindrance of substrate binding to the catalytic triad by the carbohydrate. The latter possibility suggests that glycosylation may be even more important in the catalysis of larger, more complex macromolecular protein substrates, possibly by aiding recognition of specific cleavage sites. The influence of glycosylation on the catalytic properties of MMCP-1 will not be fully understood until their native substrates are identified and characterised.

The inhibition studies of the various glyco-forms of MMCP-1 with human α_1-PI show second order association rate constants in the range 3.3 - 12.3 $x10^5$ $M^{-1}.s^{-1}$ which are similar to the 0.3 - 6.0 $x10^5$ $M^{-1}.s^{-1}$ values reported for RMCP II, trypsin and chymotrypsin with the rat serine proteinase inhibitors RS I and RS II (Pirie-Shepherd *et al* 1991). These association constants for the interaction of both rat and mouse mast cell proteases with α_1-PI inhibitors are ten-fold less than that for elastase and α_1-PI (Beatty, Bieth, & Travis, 1980) which could suggest that whilst the mast cell proteases are inhibited by α_1-PI they may not be the main target proteases for these inhibitors. On the other hand, the main plasma inhibitors of human mast cell chymase are α_1-antichymotrypsin (α_1-AC) and α_1-PI (Schechter, Sprows, Schoenberger, Lazarus, Cooperman & Rubin, 1989). The k_{ass} values for the interactions between human chymase and α_1-AC and α_1-PI are 10-fold and 100-fold, respectively less than that for the rodent chymases with α_1-PI (Schechter *et al* 1989). Similarly α_1-PI is also the main plasma inhibitor of sheep mast cell protease-1 and the

k_{ass} for this reaction is of the order of 1 x 10^3 $M^{-1}.s^{-1}$ (Pemberton, Huntley & Miller 1997). Therefore, while it is true that MMCP-1 reacts less rapidly with α_1-PI than neutrophil elastase the k_{ass} value for the reaction is still substantially greater than the rates for the mast cell chymases of other species with their homologous inhibitors, which have been demonstrated to be the main plasma inhibitors of chymases (Schechter *et al* 1989; Pemberton *et al* 1997), indicating that α_1-PI is probably the principal inhibitor of MMCP-1. The inhibition constant, K_i, for the MMCP-1 proteases is in the low pM range (7.6×10^{-10} - 2.2×10^{-9}) which is also similar to the interaction of RMCP II with the rat serpins (Pirie-Shepherd *et al* 1993). The exception to this is MMCP-1C which has a K_i of 4.5×10^{-8}, more than ten-fold greater than the others. These data clearly show that the catalytic properties of these enzymes are modified through their carbohydrate moieties with highly significant variations in how rapidly they associate with inhibitor and how tightly they are bound. This may be important in prolonging enzyme activity in situations where immediate hypersensitivity reactions have increased vascular permeability bathing surrounding tissues in serum containing high concentrations of serine proteinase inhibitors.

The rat mast cell chymase RMCP II has many similarities with MMCP-1. Both enzymes are associated with the secretory granules of mucosal mast cells and their concentrations in small intestine and in blood increase greatly during the mast cell hyperplasia associated with nematode parasite infections (Reviewed by Miller *et al* 1990). MMCP-1 and RMCP II share several physical characteristics; both are highly soluble basic proteins with net charges of +3 and +4 respectively (Huang *et al* 1991, Le Trong *et al* 1987) and share 74% amino acid sequence homology (Le Trong *et al* 1989, as well as having similar enzymic properties including very similar substrate

specificities and susceptibility to inhibitors (Reviewed by Miller *et al* 1990). Thus it is probable that MMCP-1 and RMCP II have very similar *in vivo* functions in the mouse and rat respectively. Similarly, the rat connective tissue mast cell protease RMCP I has many features with the murine CTMC protease MMCP-4 described in chapter 3. They are both highly insoluble, with similar net charge, as determined by their chromatographic properties, share 89% amino acid sequence homology and appear to be expressed exclusively in CTMC. Because of these striking biochemical similarities and because ELISA assays for both RMCP I and RMCP II have been developed in this laboratory, the studies described in the remainder of this thesis addresses the regulation of expression of RMCP I and RMCP II and their relationship to helminth infection.

5. *IN VIVO* REGULATION OF GROWTH AND PROTEASE EXPRESSION OF PERITONEAL MAST CELL POPULATIONS; EFFECTS OF EXOGENOUS STEM CELL FACTOR AND T CELL-DERIVED CYTOKINES.

5.1 Introduction

Mast cell development and survival, in rats and mice are apparently highly cytokine dependent processes with regulatory mechanisms mediated both by the T cell-derived cytokines interleukin (IL)-3, -4, -9 and -10 (Ihle, Keller, Oroszlan, Henderson, Copeland, Fitch, Prystowsky, Goldwasser, Schrader, Palaszynski & Lebel, 1983; Schmitt, Fassbender, Beyreuther, Spaeth, Schwarzkopf & Rude, 1987; Schmitt *et al* 1990; Thompson-Snipes *et al* 1991) and by the *c-kit* ligand, stem cell factor (SCF; Anderson, Lyman, Baird, Wignall, Eisenman, Rauch, March, Boswell, Gimpel & Cosman, 1990). Much of this information has been derived from *in vitro* studies but its relevance to the *in vivo* survival of mast cells and phenotypic expression of granule proteases has yet to be determined.

The mechanisms regulating rat and mouse mucosal mast cells may differ. The mast cell hyperplasia which accompanies parasitic infection is readily abrogated in the rat by treatment with corticosteroids and this is accompanied by a decrease in serum content of the mucosal mast cell protease, RMCP II (Newlands *et al* 1990). In contrast, mouse intestinal mast cell populations and serum MMCP-1 concentrations are apparently unaffected by such treatment (Newlands *et al* 1990). There are also apparent differences in the *in vivo* expression of mucosal mast cell-associated proteases in these species. In the rat, RMCP II is not only expressed in intestinal mast

cells but may also be expressed in the mast cells of lung parenchyma (Huntley *et al* 1990) and, interestingly, in peritoneal cavity mast cells (PMC) following parasitic infection (Huntley *et al* 1990b). In the mouse however, MMCP-1 is expressed almost exclusively in the mast cells of the intestinal mucosa and not in other mast cell-rich tissues such as tongue or ear pinna (chapter 3, Newlands *et al* 1993).

In vitro, rat bone marrow-derived mast cells (BMMC) develop and increase in number when cultured with IL-3 alone, whereas PMC do not (Haig *et al* 1994). In contrast, SCF supports the *in vitro* growth both of IL-3-dependent BMMC, and of isolated PMC (Tsai *et al* 1991a). However, when SCF and IL-3 are used in combination they act synergistically to promote the *in vitro* growth of both BMMC and PMC (Haig *et al* 1994). *In vivo* SCF-treatment of rats results in growth of mast cell populations in both connective tissue and mucosal locations (Tsai, Takeishi, Thompson, Langley, Zsebo, Metcalfe, Geissler & Galli, 1991) and, in the mouse, connective tissue-type mast cells proliferate in skin around the site of SCF injection (Tsai *et al* 1991b).

Protease expression *in vitro* is also regulated by cytokines. Mouse BMMC grown in IL-3 alone express MMCP-5 mRNA but the mRNAs for the mucosal mast cell proteases MMCP-1 and MMCP-2 could not be detected in Northern blots until the BMMC were exposed to IL-10 (Ghildyal *et al* 1992a; Ghildyal, Friend, Nicodemus, Austen & Stevens, 1993). Interestingly, MMCP-1 protein was detectable, by immunofluorescence and ELISA, in low concentrations, in murine BMMC grown in supernatant from the WEHI-3 cell line (Newlands *et al* 1991), a constitutive producer of IL-3 (Ihle *et al* 1983). Similarly, the serosal mast cell-associated protease

MMCP-4 mRNA was only detected in murine BMMC, developed in IL-3, when the cells were sequentially cultured or co-cultured in IL-3 and SCF (Gurish *et al* 1992), whilst a protein similar or identical to MMCP-4 was found to be co-expressed with MMCP-1 in WEHI-3-derived murine BMMC (Newlands *et al* 1991).

In the rat, the mechanism may be somewhat different since rat BMMC, cultured in IL-3 alone, express abundant RMCP II (Haig *et al* 1988) and do not express RMCP I even when co-cultured or sequentially cultured with SCF (Haig *et al* 1994). However, isolated rat peritoneal mast cells cultured in the presence of both IL-3 and SCF will synthesise and store the mucosal mast cell-associated protease RMCP II (Haig *et al* 1994).

5.2 Experimental Design

The *in vitro* data raise several important questions about the regulation of rodent mast cell growth and expression proteases *in vivo*. In this chapter the role of T cell-derived cytokines, in the form of lymph node conditioned medium, and of $rrSCF^{164}$ in regulating defined populations of mast cells; those in the peritoneal cavity and the small intestinal mucosa of the rat, will be examined.

Experiment 1.

N. brasiliensis infection in rats promotes RMCP II expression in PMC (Huntley *et al* 1990b), this experiment was therefore carried out to determine whether IL-3-rich conditioned media up-regulated the expression of RMCP II *in vivo*. To determine the *in vivo* effects of IL-3 and other T cell-derived cytokines and/or SCF on peritoneal mast cells, rats were dosed with lymph node conditioned medium (LNCM), with LNCM + SCF or SCF alone by daily intraperitoneal injection. Controls were

dosed with vehicle alone and groups of 4 rats each were killed on days 2, 4 and 7. Peritoneal mast cells were harvested by lavage. The protocols for treatment of the rats and the validation of the functional properties of the LNCM and rrSCF^{164} are detailed in Materials and Methods (2.2.3.2).

Experiment 2.

Since infection with *N. brasiliensis* promotes the expression of RMCP II in rat PMC (Huntley *et al* 1990b) the possibility that exogenously administered SCF might alter the expression of RMCP II was investigated. *Nippostrongylus*-infected and normal rats were injected daily with rrSCF^{164} in PBS, at a rate of 25 μg/kg bodyweight/day, by intravenous injection. Infected and normal controls were dosed with vehicle only and groups of treated and control rats were killed on days 8, 10 and 15. Peritoneal mast cells were harvested by lavage.

Peritoneal mast cells were also harvested from a group of normal female Wistar rats, of similar age and body weight to the experimental animals to establish normal parameters for the peritoneal mast cell population of these rats. These data are summarised in Table 10 and are referred to as baseline values throughout.

For each peritoneal lavage sample, mast cells were enumerated, protease expression was determined by immunocytochemistry and the protease content measured by ELISA.

The effects of intravenously administered rrSCF^{164} on intestinal mucosal mast cell populations was also examined in these rats.

Segments of mid-jejunum were collected and either fixed in 4% paraformaldehyde in PBS as described (2.2.8) for histological examination or stored

frozen at -20°C for subsequent ELISA assay to measure RMCP I (2.2.9) or RMCP II (2.2.10). In addition, blood samples were collected, for measurement of RMCP II by ELISA, either by tail-snip (2.2.7) or when the rats were exsanguinated.

Data are presented as median and range and were analysed by the Mann-Whitney non-parametric test. Time course data were analysed by ANOVA. The full protocols for this experiment are detailed in Materials and Methods (2.2.4).

5.3 Results

5.3.1 Effects of Intraperitoneal Injection of SCF and/or Lymph Node Conditioned Medium on Peritoneal Mast Cells.

Control groups of rats were treated with IMDM cell culture medium or IMDM plus concanavalin A at the same concentration as that present in the conditioned medium (2.2.3). There were no significant differences between these two control groups for any of the parameters tested and therefore only the data from the IMDM controls are presented here.

LNCM can generate mast cells from bone marrow precursor cells and is thus considered to be a source of the cytokines IL-3 and IL-4 (Haig, McMenamin, Gunneberg, Woodbury & Jarrett, 1983). Injection of LNCM into the peritoneal cavity caused an increase in the total number of cells recovered by peritoneal lavage when compared with the IMDM controls after 7 days of treatment ($p < 0.05$; Table 7). However there was no change in either the number of mast cells in the lavage or in the mast cell RMCP I content. RMCP II was increased in the peritoneal mast cells as early as day 2 of treatment and showed a > 11-fold increase compared with IMDM

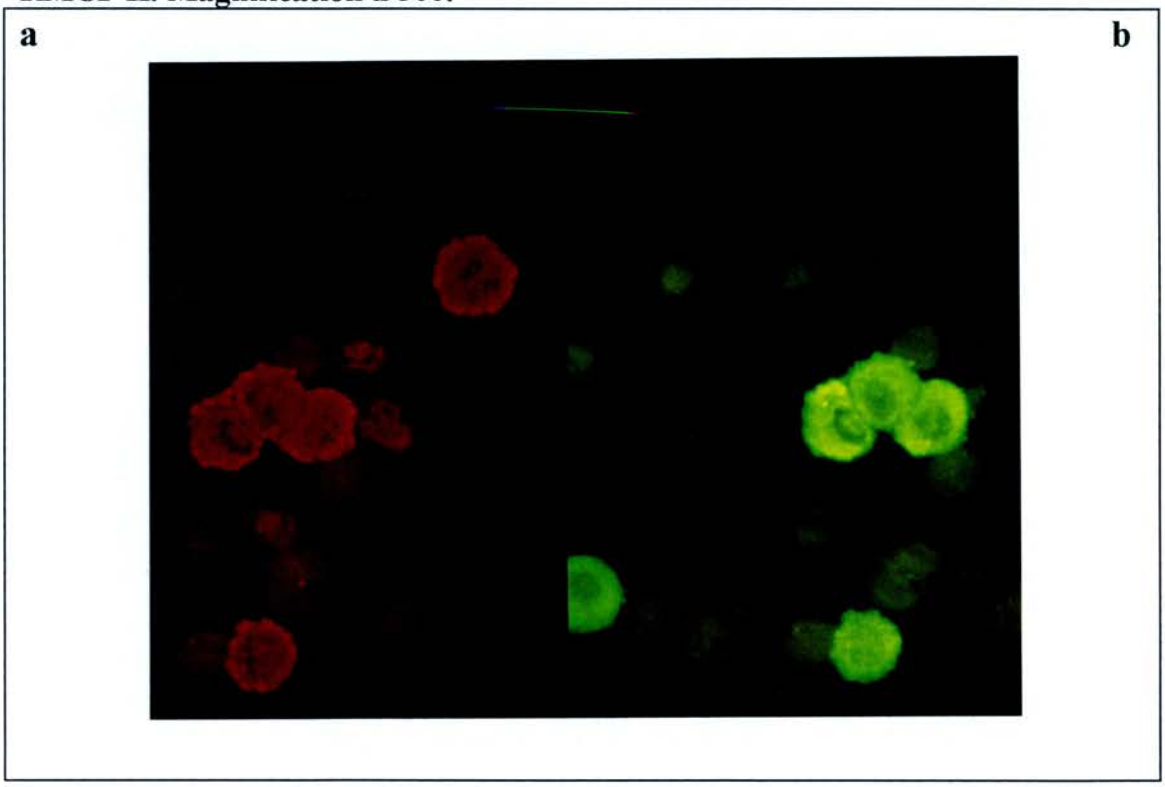
controls ($p < 0.05$) on day 7 (Table 7). The increase in RMCP II content was also reflected in the proportion of mast cells which stained for the presence of RMCP II, by immunofluorescence, which was significantly increased on both days 4 and 7 of treatment compared with controls (Table 7, $p < 0.05$ for both).

Table 7. Effects of intraperitoneal injection of SCF and/or lymph node conditioned medium on peritoneal mast cell populations.

	Day	n	Total Cells/Lavage ($x10^7$)	Total Mast Cells/Lavage ($x10^6$)	RMCP I (pg/Mast Cell)	RMCP II (fg/Mast Cell)	% RMCP II^+ Mast Cells
LNCM only	2	4	2.7 (2.3 - 3.3)	1.2 (1.0 - 1.4)	96.1 (72 -185)	64 (36 - 131)	3.6 (1.5 - 4.3)
	4	4	2.7 (1.9 - 3.6)	0.9 (0.7 - 1.2)	118 (95 - 179)	122* (57 - 321)	11* (5 - 17)
	7	4	4.2* (1.8 - 3.9)	1.4 (0.9 - 1.9)	90 (68 - 115)	302* (236 - 418)	22* (13 - 25)
LNCM + SCF	2	4	2.0 (1.8 - 2.3)	0.7* (0.6 - 0.8)	130 (119 - 130)	57* (39 - 73)	1.5 (0.5 - 2.4)
	4	4	2.4 (1.0 - 3.6)	1.6 (0.3 - 2.3)	65 (42 - 95)	271* (134 - 435)	17* (10 - 18)
	7	4	4.4* (3.1 - 5.6)	5.6* (2.4 - 8.5)	34 (27 - 65)	325* (210 - 576)	30* (24 - 38)
SCF only	2	4	1.8 (1.5 - 2.4)	1.0 (0.9 - 1.2)	103 (76 - 136)	20 (7 - 37)	1 (0 - 2)
	4	4	1.5* (1.3 - 2.3)	1.4 (1.2 - 2.1)	63 (59 - 94)	52 (36 - 79)	10* (7 - 11)
	7	4	2.3 (1.8 - 3.9)	3.4* (2.0 - 3.8)	38* (27 - 47	85* (58 - 251)	7* (2 - 16)
Medium only control	7	4	2.3 (1.6 - 2.9)	0.9 (0.8 - 1.2)	137 (46 - 227)	28 (18 - 37)	1.5 (0.5 - 2.4)
ANOVA			$p = 0.001$	NS	NS	$p = 0.001$	$p < 0.001$

Data are presented as median and range. For each parameter the data were analysed by one way analysis of variance to determine whether there were differences between the treatment groups. The data were further analysed by Mann-Whitney non-parametric test where each group was compared with the medium only control; * = $p < 0.05$.

Figure 11. Photomicrograph of peritoneal mast cells from rats treated with LNCM + SCF and dual labelled for the presence of RMCP I and RMCP II by immunofluorescence.
Cytocentrifuge preparation of peritoneal lavage cells stained with a) rabbit anti-RMCP I and visualised with TRITC-anti-rabbit conjugate and b) the same preparation concomitantly stained with FITC-conjugated monoclonal anti-RMCP II. Magnification x 300.



When rats were treated with a combination of both LNCM and SCF there was a slight increase in the total number of cells recovered by lavage at day 7 compared with controls ($p > 0.05$, Table 7). In contrast with the LNCM-treated rats, those which received both LNCM and SCF showed an initial decrease in the number of mast cells recovered at day 2, compared with controls ($p < 0.05$), before increasing at day 7, where there were 5.5-fold more mast cells than were present in the controls (Table 7). The growth in mast cell numbers was followed by a decline in their RMCP I content to 34 pg/cell compared with 137 pg/mast cell in the controls ($p > 0.05$; Table 7).

Unlike RMCP I, the RMCP II content of the mast cells from the LNCM + SCF-treated group was increased by 2-fold at day 2 compared with controls ($p < 0.05$; Table 7) and continued to rise throughout the course of the experiment until the RMCP II content was almost 13-fold greater than controls at day 7 ($p < 0.05$; Table 7). The rise in RMCP II content, as measured by ELISA, was again reflected in the percentage of mast cells which stained for the presence of RMCP II by immunofluorescence; rising to 30% of all mast cells showing the RMCP I^+/II^+ phenotype by day 7 of the experiment (Figure 11, Table 7, $p < 0.05$).

The group which were treated with SCF alone did not exhibit any overall increase in peritoneal cell numbers compared with IMDM-treated controls, indeed there was a significant reduction in total cell numbers at day 4 ($p < 0.05$; Table 7). There was, however, an increase in mast cell numbers at both days 4 and 7 compared with controls ($p < 0.05$ on day 7; Table 7), similar to the LNCM + SCF-treated group. There was also a decrease in the RMCP I content of these mast cells; by as much as 75% on day 7 compared with controls ($p < 0.05$; Table 7). Like the groups treated with LNCM alone or LNCM + SCF, the group treated with SCF alone showed an increase in the RMCP II content of their serosal mast cells, when compared with controls, although to a lesser extent than that seen in the other groups ($p = 0.001$ by ANOVA, Table 7). Similarly the RMCP II immunofluorescence showed a significantly increased proportion of the serosal mast cells were RMCP II^+ at both days 4 and 7 compared with controls ($p < 0.05$ for both) but fewer than either the LNCM alone of LNCM + SCF-treated groups (Table 7).

5.3.2 Intravenous injection of rrSCF^{164} and infection with N. brasiliensis*; Effects on peritoneal mast cell populations.*

Administration of SCF alone for 15 days caused a 5-fold increase in the number of mast cells present in the peritoneal lavage ($p < 0.0001$, Table 8, Figure 12a and b) when compared with rats treated with vehicle only, while no difference was noted in the total number of cells recovered by lavage from either group (Table 8). The increase in the mast cell population was accompanied by a significant ($p < 0.01$) reduction in the mean peritoneal mast cell RMCP I content from 26 to 17 pg/mast cell (Table 8). Only trace amounts of the mucosal mast cell-associated protease, RMCP II was detected in the peritoneal lavage cell pellet (Table 8) and none of the cells stained for the presence of RMCP II by immunofluorescence (Table 8).

Infection with *N. brasiliensis* significantly depleted the number of mast cells in the peritoneal cavity over the course of the infection when compared with the day 15 vehicle-treated controls ($p < 0.05$ for days 8 and 15 and $p < 0.01$ for day 10 of infection) while no reduction was seen in the peritoneal cell population in general (Table 8). SCF-treated rats infected with *N. brasiliensis,* on the other hand, showed no corresponding decrease in mast cell numbers at days 8 and 10 and a slight increase at day 15 of infection/treatment, when compared with vehicle-treated controls, although this did not achieve statistical significance (Table 8). This was accompanied by a general increase in the peritoneal cell population when compared with vehicle-treated controls ($p < 0.05$; Table 8). Comparison of the mast cell numbers, over the course of infection with *N. brasiliensis,* for the two treatments, showed a highly significant difference between infected animals treated with vehicle alone and those treated with SCF ($p < 0.0005$ by ANOVA; Table 8).

In *N. brasiliensis*-infected rats the RMCP I content of the PMC was somewhat increased during infection when compared with vehicle-treated controls (Table 8). However these values were still significantly lower than baseline values, from normal animals, on days 8 and 15 ($p < 0.01$ for each; Table 8, Table 10). There was no increase noted in the PMC content of RMCP I in infected animals treated with SCF when compared with medium only-treated controls, but again, these values were significantly lower than baseline values (Table 8, Table 10). Comparison of the RMCP I content of the PMC from SCF-treated and -untreated rats over the course of the *N. brasiliensis* infection showed that there was significantly less enzyme in the SCF-treated rats ($p = 0.006$ by ANOVA; Table 8).

The most striking differences between SCF-treated and -untreated rats were in the expression of RMCP II, a mucosal mast cell-associated protease, in what are regarded as the typical connective tissue mast cells of the peritoneal cavity. Vehicle-treated control rats contained no or very little RMCP II in the peritoneal cavity, with 0 - 8 fg/mast cell being detected. This was not significantly altered by treatment with rrSCF[164]. The RMCP II content of the serosal mast cells of *N. brasiliensis*-infected rats was significantly increased by day 8 of infection ($p < 0.05$) and reached a peak at day 10 and although still increased, when compared with vehicle-treated controls, at day 15 (Table 8) the RMCP II content had begun to decrease. This effect was even greater in the infected animals treated with SCF where the RMCP II content of the PMC was increased to 86 fg/cell at day 8 and continued to increase to over 5000 fg/cell at day 15 of infection (Table 8).

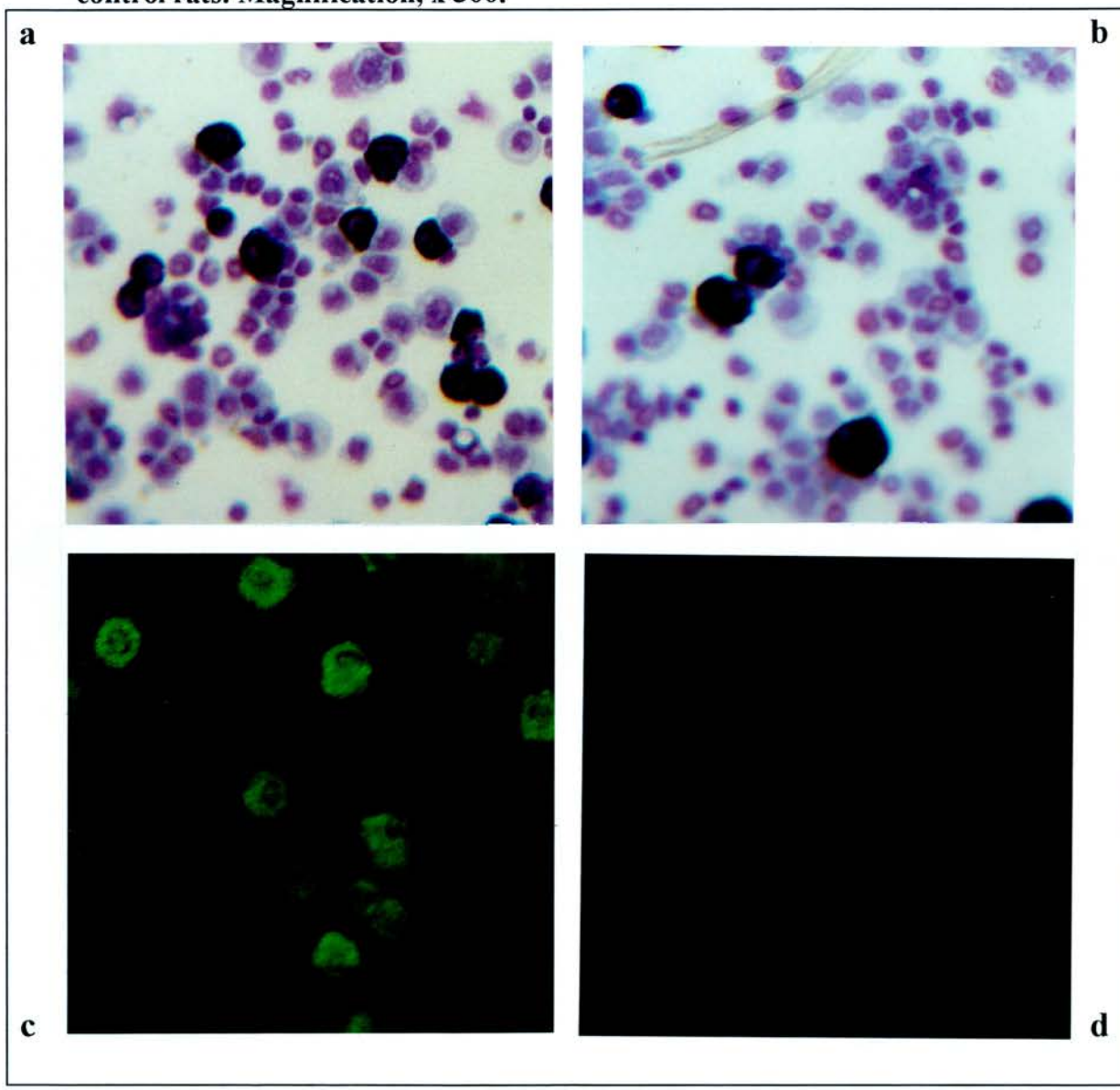
Table 8. The effects of intravenous injection of SCF on peritoneal mast cell populations in normal and parasitised rats.

	n	Total Cells/Lavage ($x10^7$)	Total Mast Cells/Lavage ($x10^6$)	RMCP I (pg/Mast Cell)	RMCP II (fg/Mast Cell)	% RMCP II^+ Mast Cells
D 8 Nb only	3	2.0	0.2*	79	20*	80
		(2.0 - 2.9)	(0.1 - 0.3)	(52 - 98)	(20 - 23)	(64 - 100)
D 10 Nb only	4	3.6	0.3**	114	355*	100
		(0.9 - 14.0)	(0.2 - 0.7)	(92 - 221)	(25 - 2820)	(100 - 100)
D 15 Nb only	4	2.6	0.6*	83	49*	28
		(2.1 - 5.4)	(0.1 - 1.0)	(39 - 108)	(13 - 8080)	(0 - 100)
D 8 Nb-SCF	3	2.2	1.6	36	86*	96
		(1.7 - 2.9)	(1.1 - 2.2)	(28 - 51)	(73 - 158)	(92 - 100)
D 10 Nb-SCF	4	2.4	2.2	51	218*	25
		(1.9 - 3.1)	(1.2 - 3.0)	(23 - 95)	(115 - 7390)	(14 - 100)
D 15 Nb-SCF	7	3.5	4.5	33	5050**	69
		(1.6 - 5.8)	(0.5 - 6.2)	(25 - 44)	(45 - 10800)	(0.8 - 100)
D 15 SCF only	8	2.3	7.7***	17** (n=5)	2 (n=5)	0
		(1.6 - 2.5)	(6.4 - 8.9)	(16 - 20)	(0 - 10)	(0 - 0)
D 15 medium	7	2.1	1.6	26 (n=5)	0 (n=5)	0
only control		(1.7 - 3.0)	(0.7 - 2.2)	(23 - 78)	(0 - 8)	(0 - 0)
ANOVA		NS	$p < 0.0005$	$p = 0.006$	NS	NS

Data are presented as median and range. For each parameter the data from each treatment group were analysed by one way analysis of variance to determine whether there were differences between the treatments. The data were further analysed by the Mann-Whitney non-parametric test where each group was compared with the appropriate medium only control; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

However, the difference in RMCP II content between SCF-treated and - untreated rats over the course of infection was not statistically significant. These marked increases in RMCP II content were reflected in the immunohistochemical staining of cyto-smears for the presence of RMCP II (Figure 12c).

Figure 12, Photomicrographs of peritoneal lavage cells from rats treated with rrSCF^{164} for 15 days or from vehicle-only-treated controls.
Toluidine blue stained cytocentrifuge preparations from a) rrSCF^{164}-treated or b) vehicle-only-treated control rats. FITC-labelled anti-RMCP II-stained cytocentrifuge preparations from c) rrSCF^{164}-treated or d) vehicle-only-treated control rats. Magnification, x 300.



There were no RMCP II$^+$ mast cells detected in cyto-smear preparations from vehicle only-treated controls or from rats treated with SCF alone (Table 8, Figure 12d), although mast cells in preparations from both these groups stained strongly for the presence of RMCP I. In rats infected with *N. brasiliensis* 80% of the mast cells in cyto-smear preparations were RMCP II$^+$ by day 8 of infection, rising to 100% by day 10, before falling again to < 30% on day 15 (Table 8). Mast cells in infected, SCF-treated animals showed maximum fluorescence earlier than those in the vehicle-treated group, at day 8 of infection, with 96% of all mast cells staining for the presence of RMCP II (Table 8). The fluorescence thereafter decreased to levels similar to that seen in the vehicle-treated, *N. brasiliensis*-infected group (Table 8). Over the course of *N. brasiliensis* infection there was no significant difference in the percentage of mast cells staining for the presence of RMCP II between SCF-treated and -untreated rats.

The peritoneal mast cells of the control group, treated for 15 days with vehicle alone, had similar values to those of the baseline group (Table 8) for all the parameters measured except that their RMCP I content was significantly lower at < 36 pg/cell compared with a baseline value of 143 pg/mast cell (Table 8, Table 10).

5.3.3 Effects of rrSCF^{164} on jejunal MMC numbers and mast cell-associated proteases in N. brasiliensis*-infected rats*

Infection with *N. brasiliensis* causes an initial depletion of MMC from the jejunum of the rat followed subsequently by substantial MMC hyperplasia. The data in Table 9 confirm that mast cells were completely depleted from the jejunum at day 8 of infection (n = 3) compared with control values in non-infected rats (n = 5, $p < 0.05$;

Table 9). By day 15 of infection mast cell densities in jejunum were increased nine-fold over control values (Table 9). The concentrations of RMCP II reflect the MMC counts in the jejunum with a 5.8 fold increase on day 15 when compared with control values in rats not treated with $rrSCF^{164}$.

Treatment of rats with $rrSCF^{164}$ for 15 days, resulted in a significant (~85%; $p < 0.001$) elevation in mean jejunal mast cell density when compared with untreated controls, a result which is in good agreement with previously published values (Tsai *et al* 1991a). The mast cell densities at 8, 10 or 15 days of *N. brasiliensis* infection in rrSCF-treated or -untreated animals were not significantly different. There were more mast cells at day 8 in specimens from $rrSCF^{164}$-treated vs. -untreated rats, but with a group size of 3 this difference was not significant. On the other hand, the large difference in RMCP II content on day 8 between $rrSCF^{164}$-treated and -untreated parasitised rats is significant ($p < 0.05$; Table 9).

Data for RMCP I content confirm previous findings and show that the concentration of RMCP I in the jejunum is several orders of magnitude less than that of RMCP II and these values change to a lesser extent than values for RMCP II as a result of *N. brasiliensis* infection (Table 9).

Table 9 Effects of SCF treatment on jejunal mast cell density and mast cell protease content in rats infected with *N. brasiliensis.*

	SCF	Control (not infected)	Day of infection		
			8	10	15
		n = 5	n = 3	n = 5	n = 7
Mast cells /0.2 mm^2	-	26 ± 1.2	0.0 ± 0	72 ± 16	239 ± 27
	+	49 ± 4.2***	13 ± 6.8	69 ± 25	221 ± 4
RMCP I (μg/gram)	-	3.0 ± 0.9	1.0 ± 0.4	1.0 ± 0.2	7.0 ± 2.3
	+	6.0 ± 1.5	2.0 ± 0.4	4.0 ± 0.9	20 ± 5.8
RMCP II (μg/gram)	-	426 ± 59	19 ± 4.7	423 ± 181	2464 ± 607
	+	470 ± 60	375 ± 79*	776 ± 281	8711 ± 3839

* = $p < 0.05$, *** = $p < 0.001$ by two-tailed Student's t-test versus value for SCF-untreated animals at the same time point.

5.3.4 Effects of rrSCF^{164} on systemic secretion of RMCP II during infection.

Daily intravenous injection of rrSCF^{164} had no effect on the systemic secretion of RMCP II in control (uninfected) rats in which serum values remained at ≅ 300 ng/ml in both SCF-treated and -untreated controls throughout the course of the experiment (Figure 13). The concentration of RMCP II increased to 2350 ng/ml in the control parasitised group on day 10 of infection but the peak serum RMCP II values occurred earlier, on day 8, in the rrSCF^{164}-treated group. The day 8 value in the SCF-treated group (4650 ng/ml) was significantly higher than the corresponding value (1670 ng/ml) in the SCF-untreated rats (Figure 13, n = 5, $p < 0.05$,). Together with the data in Table 9, this result shows that at day 8 of infection, rrSCF^{164} treatment results in both increased jejunal RMCP II content and increased secretion of

this mast cell protease. Moreover, by ANOVA, serum levels of RMCP II over the entire time course of the infection were significantly higher in the rrSCF^{164}-treated than in the control group not treated with the cytokine ($p = 0.001$).

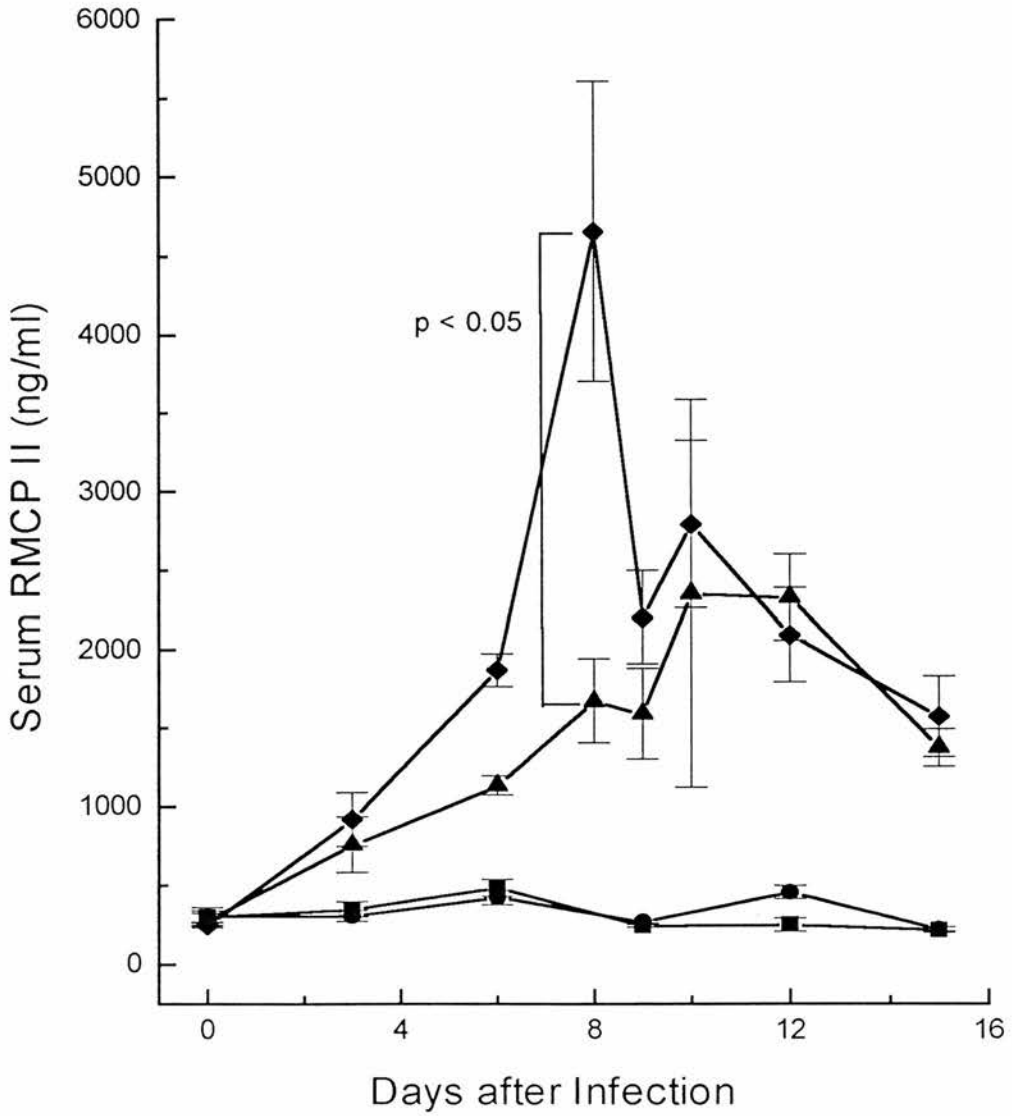


Figure 13, Serum concentrations of RMCP II in rats treated with rrSCF^{164}. Comparison of uninfected animals treated with rrSCF^{164} (●) or vehicle alone (■) and *N. brasiliensis*-infected animals treated with rrSCF^{164} (◆) or vehicle controls (▲).

5.3.5 Effect of rrSCF^{164} on parasite fecundity.

The effect of the administration of rrSCF^{164} on the fecundity of *N. brasiliensis* was determined by monitoring egg output in the faeces of the rats. Egg output was maximal on day 6 of infection in both rrSCF^{164}-treated and vehicle-treated (control) rats and diminished until no eggs were detected by day 11 in either group (Figure 14). While values for faecal egg output were greater in the rrSCF^{164}-treated as opposed to control rats at many intervals of the infection, these differences did not achieve statistical significance.

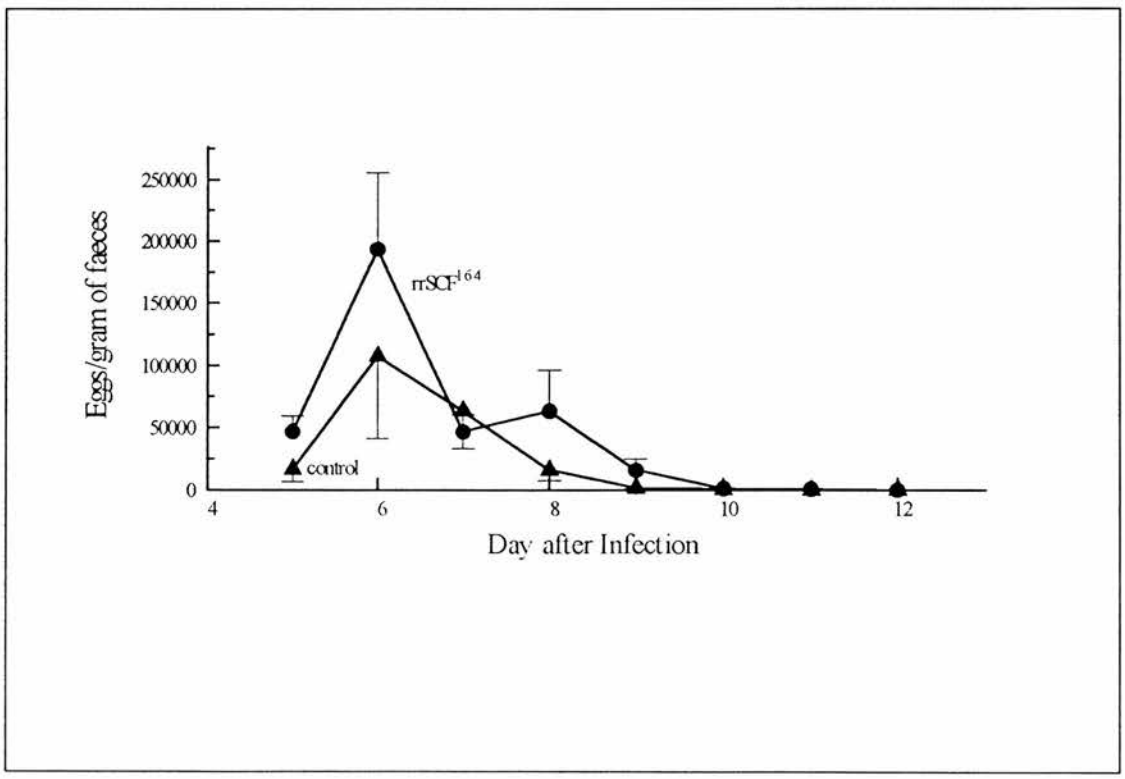


Figure 14, Faecal egg counts from *N. brasiliensis*- infected rats treated with rrSCF^{164}.

Table 10; Baseline Peritoneal Mast Cell Parameters for a Group of Normal Rats.

n	Total Cells/Lavage ($x10^7$)	Mast Cells/Lavage ($x10^6$)	RMCP I (pg/Mast Cell)	RMCP II (fg/Mast Cell)	% RMCP II^+ Mast Cells
5	1.9 ± 0.12	0.8 ± 0.07	143 ± 8.1	6 ± 1.0	0 ± 0.0

5.4 Discussion

The increase in numbers of PMC after intravenous treatment with SCF confirms the previous *in vivo* and *in vitro* findings that SCF is a potent growth factor for PMC (Tsai *et al* 1991a, Haig *et al* 1994). However, unlike the previous studies which showed the concentrations of the PMC-associated protease RMCP I to be maintained (Haig *et al* 1994) or increased (Tsai *et al* 1991a), the results described here show the RMCP I content of the peritoneal mast cells to be decreased by about 50% compared with controls. This raises the possibility that, while SCF provides a potent proliferative and/or recruitment signal for PMC it does not stimulate, and may even down-regulate synthesis and/or storage of RMCP I.

Following infection with the intestinal nematode parasite *N. brasiliensis* the mast cell population of the peritoneal cavity was greatly reduced (Table 8), possibly as a result of degranulation (Wilson & Bloch 1968), although the mean RMCP I content of the remaining mast cells was increased compared with vehicle treated controls. These data demonstrate that infection has profound effects on mast cell populations in locations remote from the site of infection. The depletion of peritoneal mast cells was accompanied by a substantial increase in the RMCP II content of the remaining mast cells as measured by both ELISA and immunofluorescence. A similar

result was obtained when *N. brasiliensis*-infected rats were treated with SCF, except that the induction of the peritoneal RMCP II response was even greater in the SCF-treated animals (Table 8). These results are in accord with the previously reported finding that IL-3 and SCF act in synergy not only to stimulate mast cell growth but also to cause a sub-population of serosal mast cells, *in vitro*, to synthesise and store RMCP II (Haig *et al* 1994). The intestinal mastocytosis associated with nematode infection involves the activities of both the T cell-derived cytokines IL-3 and IL-4 (Madden *et al* 1991) and the connective tissue-derived factor SCF and its receptor, *c-kit* (Grencis *et al* 1993, Newlands *et al* 1995) and thus it is probable that these cytokines also have synergistic effects *in vivo* which lead to the induction of RMCP II synthesis by serosal mast cells. Groups of rats treated intraperitoneally with LNCM or SCF either singly or in combination confirmed the results from the parasitic model. Treatment with the cytokine-rich LNCM did not cause any expansion of the serosal mast cell population and did not alter the mean mast cell RMCP I content but it did stimulate increased synthesis of RMCP II. Treatment with SCF alone caused an increase in PMC numbers coupled with a decrease in RMCP I content and, surprisingly, an increase in RMCP II content. This increase in RMCP II may be due to either a synergistic effect of the rrSCF with background levels of IL-3 or, perhaps to some effect of the rrSCF being infused directly into the peritoneal cavity, such as greater local concentration, than in the systemic treatment. Combination of LNCM and SCF substantially increased mast cell numbers but again the presence of SCF depressed the mean mast cell RMCP I content. This is in contrast with *in vitro* murine BMMC which showed an increase in mRNA for the PMC-associated chymase

MMCP-4, the mouse analogue of RMCP I, when sequentially cultured or co-cultured in IL-3 and SCF (Gurish *et al* 1992).

In confirmation of previous reports (Tsai *et al* 1991a), treatment of uninfected rats with rrSCF^{164} at 25 μg/kg/day, i.v. for 14 days produced a significant (85%) increase in jejunal MMC numbers. However, the elevation in jejunal RMCP II content in rrSCF^{164}-treated vs. vehicle-treated rats did not achieve statistical significance. On the other hand, rrSCF^{164} treatment had only modest effects on the changes in jejunal MMC numbers, or on the jejunal or serum levels of RMCP II, that were associated with *N. brasiliensis* infection. Compared to vehicle-treated rats, rrSCF^{164}-treated rats had higher levels of jejunal MMCs and significantly higher levels of jejunal RMCP II at day 8 of infection. Also, the peak mean levels of serum RMCP II occurred earlier (day 8 vs. day 10) and reached a roughly 2-fold higher maximum in rrSCF^{164}-vs. vehicle-treated rats. These findings indicate that administration of exogenous SCF can alter the kinetics and increase the magnitude of jejunal MMC development and RMCP II production during *N. brasiliensis* infection. However, later during infection, numbers of jejunal MMCs and serum levels of RMCP II were quite similar in rrSCF^{164} and vehicle-treated rats, and, due to the broad range of individual values, the higher mean levels of RMCP II in the jejunal mucosa of rrSCF^{164}-treated rats were not significantly different than those in the vehicle-treated controls. One possible explanation for these findings, is that endogenous levels of SCF in normal rats are sufficient to permit near maximal MMC hyperplasia (presumably in response to T cell-associated cytokines) in the setting of *N. brasiliensis* infection.

Taken together these two experiments clearly show SCF to provide a potent stimulus for PMC growth and/or recruitment while depressing RMCP I expression and/or storage. Furthermore, intravenous treatment with SCF did not up-regulate RMCP II expression whereas direct delivery of SCF into the peritoneal cavity did. This could be due to SCF-induced secretion of IL-3 by the resident population of mast cells.

In summary, SCF causes significant proliferation and/or recruitment of connective tissue mast cells in the peritoneal cavity of the rat and that SCF or LNCM stimulate the up-regulation of RMCP II expression in these cells. This up-regulation of RMCP II expression is even greater if SCF and LNCM are used in combination than if either cytokine is used individually.

6. ROLE OF STEM CELL FACTOR IN SUPPORTING MAST CELL SUB-SETS IN NORMAL RATS

6.1 Introduction

In chapter 5 the effects of exogenous cytokines and growth factors on peritoneal mast cells were investigated. In this chapter these studies will be extended to examine the effects of depleting SCF by treatment with antibodies. Mice or rats with mutations which markedly diminish *c-kit* tyrosine kinase activity such as W/W^v mice (Reed 1989) or W^s/W^s rats (Arizono *et al* 1993) develop no or very few mast cells in comparison with normal animals. Moreover, treatment of *T. spiralis*-infected mice with antibodies to *c-kit* blocked the intestinal mast cell hyperplasia associated with parasitic infection (Grencis *et al* 1993). *In vitro,* SCF supports the survival and growth of both rat bone marrow-derived mast cells, which have many of the features of MMCs, and peritoneal cavity derived CTMC (Haig *et al* 1994). SCF also promotes the survival, both *in vivo* and *in vitro*, of IL-3 dependent mast cells which otherwise undergo apoptosis when they are deprived of IL-3 (Iemura, Tsai, Ando, Wershil & Galli, 1994). Thus, from this evidence, it is clear that SCF and *c-kit* play an important role in promoting mast cell growth *in vitro,* in supporting IL-3 dependent mast cells and in gastrointestinal nematodiasis-associated mast cell hyperplasia. There is, however, little or no data on the regulatory role of SCF *in vivo* on resting mast cell populations in normal animals. To address this question rats were treated with polyclonal antibody to SCF to determine what the consequences of blocking these activities would be for the mucosal and peritoneal mast cell populations.

6.2 Experimental Design

Aim:- to determine whether mast cell populations in normal rats are SCF-dependent. The approach taken to investigate the role of SCF in regulating mast cell populations was to attempt to block its function by treating rats with polyclonal antibodies. However, since SCF has important roles in regulating stem cell haemopoiesis the effect of such treatment on various haematological parameters was also monitored.

Protocol 1:

To determine the effects of treatment with anti-SCF antibodies (2.2.6) on mast cell populations and protease expression, rats were treated with antibody for 4 or 7 days and killed 24 hours after the final inoculation. Samples were collected by peritoneal lavage for cytosmear and cell pellet preparation. Samples of small intestine, stomach, lung, tongue and liver were collected into fixative for histology (2.2.8) or were frozen for subsequent measurement of mast cell protease concentrations by ELISA (2.2.9, 2.2.10). Mast cells were enumerated in lavage samples and histological sections and protease phenotypes determined by immunohistochemical localisation of mast cell proteases. Serum samples were also collected and assayed for the MMC-associated protease RMCP II (2.2.10).

Protocol 2:

To determine the effects of anti-SCF treatment on haematological parameters a second experiment was carried out where rats were treated for 4 days with anti-SCF antibodies or normal IgG. Blood samples were collected from all rats into anti-

coagulant (2.2.7) at the start of the experiment, on day 0, to establish normal haematological parameters and again at the termination of the experiment on day 5 to determine the impact that these treatments had on haemopoiesis. The details of these protocols are described in Materials and Methods (section 2.2.7).

6.3 Results

6.3.1 Effects of anti-SCF antibody treatment on peritoneal cavity mast cells.

Treatment of normal rats with polyclonal sheep antibodies to rrSCF had a marked effect on the peritoneal mast cell population. After 4 days of treatment the numbers of mast cells recovered by peritoneal lavage had decreased by 58% ($p < 0.05$; Table 11) compared with normal sheep IgG-treated (control) animals and the total PMC-associated protease, RMCP I, in the cell pellet had decreased by 62% ($p < 0.01$; Table 11) compared with controls. At the same time the mean RMCP I content per mast cell was decreased but this did not achieve statistical significance. When the antibody treatment was continued for 7 days mast cell numbers decreased by 55% compared with controls ($p < 0.05$; Table 11), a similar result to that recorded after 4 days of antibody treatment, whilst the RMCP I content of the cell pellet decreased by 47% of control values although, again, this did not achieve statistical significance. The mean mast cell RMCP I content, after 7 days of antibody treatment, showed an apparent increase of 86% over control values (Table 11) but this was also not statistically significant and was probably due to the decrease in control values, from day 4 to day 7 in the mast cell RMCP I content. This was in contrast with the total number of cells recovered by peritoneal lavage which showed no decrease after 4 days of antibody treatment and only a 10% decrease after 7 days, although neither value

was statistically significant. As there was no greater depletion of mast cells or RMCP I from the peritoneal cavity after 7 days of antibody treatment compared with 4 days of treatment further analysis was confined to material collected from the 4 day treatment experiment.

Table 11 Mast cell numbers, total cell numbers and total RMCP I levels from peritoneal lavage cell pellets recovered from rats treated with polyclonal sheep anti-SCF or normal sheep IgG (control) for 4 or 7 days.

days of treatment		4				7			
	n	Total cell No. ($\times 10^7$)	Mast cell No. ($\times 10^5$)	RMCP I (μg/lavage)	RMCP I (pg/mast cell)	Total cell No. ($\times 10^7$)	Mast cell No. ($\times 10^5$)	RMCP I (μg/lavage)	RMCP I (pg/mast cell)
Anti-SCF	5	2.6 ± 0.22	2.2 ± 0.57*	34.3 ± 15.12**	137 ± 34.3	2.8 ± 0.28	3.1 ± 1.5*	37.3 ± 8.53	205 ± 64.5
control	5	2.6 ± 0.26	5.1 ± 0.96	97.7 ± 9.58	212 ± 27.3	3.1 ± 0.19	6.8 ± 0.97	69.8 ± 16.4	110 ± 6.8
% decrease		0%	58%	62%	35%	10%	55%	47%	(86% increase)

Student's two tailed t-test; * = $p < 0.05$, ** = $p < 0.01$

There is a strong correlation between peritoneal mast cell numbers, regarded as typical CTMC, and RMCP I content (Figure 15, $r = 0.82$, $p = 0.0067$) and therefore measurement of RMCP I by ELISA was used to assess the systemic effect of anti-SCF treatment on the CTMC populations in other anatomical sites.

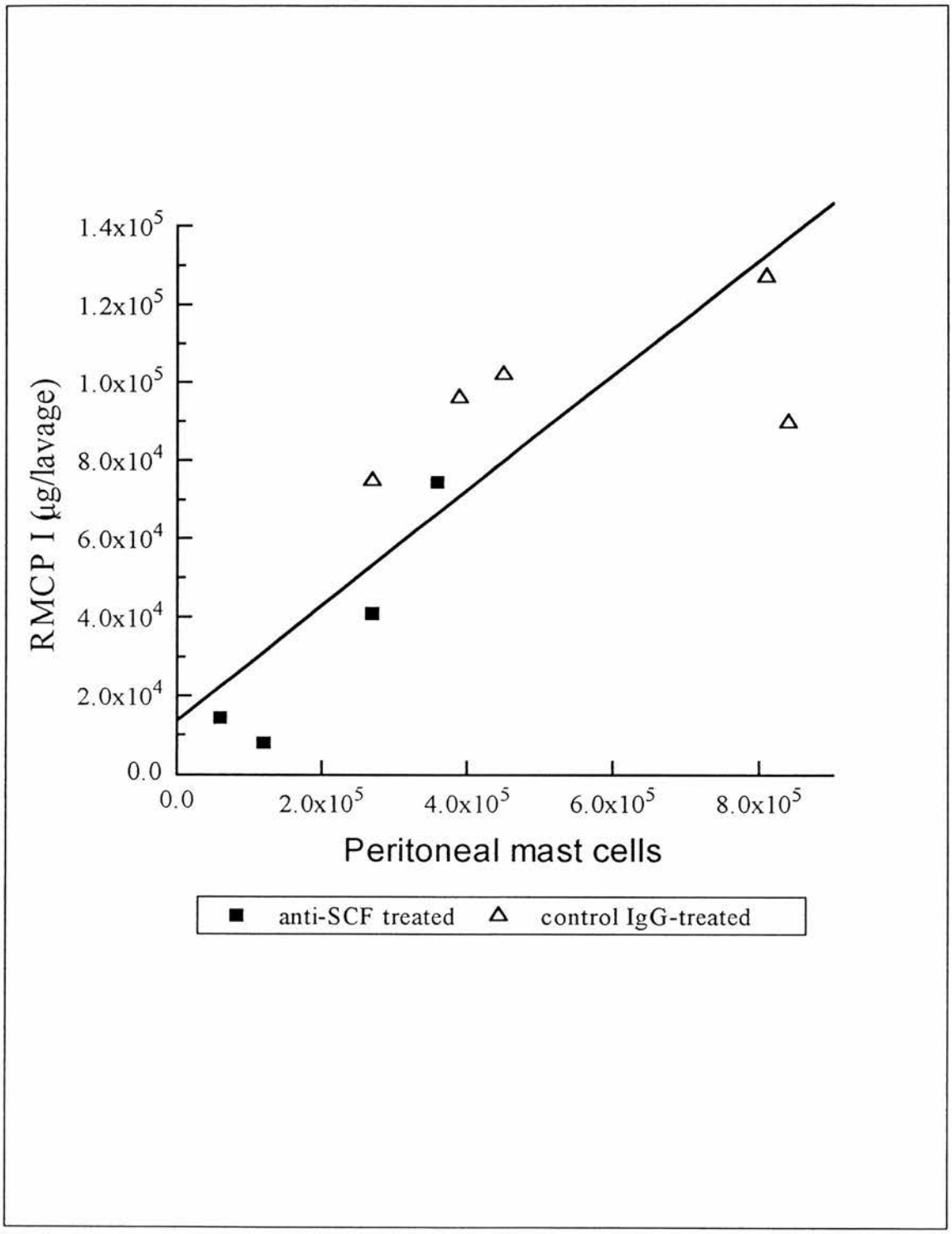


Figure 15 Graph showing a plot of peritoneal mast cell number against total RMCP I for peritoneal lavage samples from rats treated with anti-SCF antibodies or with normal sheep IgG.

6.3.2 Effects of anti-SCF treatment on RMCP I concentrations in other organs.

RMCP I concentrations in stomach and small intestine were depleted by 60% and 65% respectively ($p < 0.05$ for each, Table 12) which was similar to the level of depletion from the peritoneal cavity. There was an even greater depletion from liver (80%, $p < 0.001$, Table 12) but only a 36% decrease in the RMCP I concentration in tongue although this was significant ($p < 0.05$, Table 12). Interestingly the smallest depletion of RMCP I was from lung where protease concentrations decreased by only 20% compared with control values ($p > 0.05$).

Table 12 RMCP I levels, measured by ELISA, in several tissues from rats after 4 days treatment with sheep anti-SCF or normal sheep IgG (control) antibodies.

	Small Intestine	Lung	Tongue	Stomach	Liver
Anti-SCF	0.25 ± 0.08*	8.8 ± 0.14	61.8 ± 12.0*	2.9 ± 0.96*	0.24 ± 0.07***
control	0.7 ± 0.02	11.0 ± 1.2	96.0 ± 6.96	7.2 ± 1.3	1.2 ± 0.10
% decrease	65%	20%	36%	60%	80%

The results are expressed as mean ± sem in μg/g wet wt. of tissue

Student's two tailed t-test; * = $p < 0.05$, *** = $p < 0.001$

6.3.3 Effects of anti-SCF treatment on mucosal mast cells and RMCP II levels.

Anti-SCF treatment had a more pronounced effect on the intestinal MMC population than on CTMC in the peritoneal cavity or in other organs. After 4 days of treatment the intestinal MMCs were totally ablated with no mast cells being detected in toluidine blue stained sections of jejunum ($p < 0.001$, Table 13). This was reflected in the amounts of RMCP II detected in jejunal extracts and in serum by ELISA where

> 95% reduction was recorded for both ($p < 0.01$, Table 14). There was a less marked effect in stomach, for while there was a 73% reduction in mast cell density in the gastric mucosa compared with control values ($p < 0.05$, Table 13), there was only 34% decrease in RMCP II content ($p > 0.05$). Concentrations of RMCP II in liver were also decreased by 82.5% in anti-SCF-treated animals when compared with normal IgG-treated (control) animals ($p < 0.01$, Table 14) which is similar to the level of decrease noted in the RMCP I content of liver (Table 12). Despite an apparently large decrease in the RMCP II content of lung (Table 14) the intra-group variability precluded statistical significance.

Table 13 Mucosal mast cell counts from toluidine blue stained sections of small intestine or stomach from rats treated with anti-SCF or normal sheep IgG (control) antibodies.

	small intestine	stomach
Anti-SCF	0.0 ± 0.0***	5.8 ± 4.1*
control	6.5 ± 1.3	21.4 ± 4.6
% decrease	100%	73%

Results are mean ± sem mast cells/0.2 mm^2

Mann-Whitney non-parametric test * = $p < 0.05$, *** = $p < 0.001$

Table 14 RMCP II concentrations in tissue extracts and serum from rats treated for 4 days with sheep anti-SCF or normal sheep IgG (control) antibodies.

	lung	liver	stomach	Small Intestine	serum (ng/ml)
anti-SCF	5.0 ± 1.22	0.3 ± 0.05**	22.6 ± 5.24	0.4 ± 0.25**	7.0 ± 1.92**
control	17.7 ± 7.94	1.6 ± 0.60	34.2 ± 9.17	342.8 ± 45.07	157.0 ± 15.27
% decrease	72.0%	82.5%	34.1%	99.9%	95.6%

Results are mean ± sem μg/g wet wt. of tissue or ng/ml of serum.

Mann-Whitney non-parametric test ** = $p < 0.01$

6.3.4 Effects of anti-SCF treatment on haematological parameters.

Stem cell factor is fundamentally involved in haemopoiesis since mice with a mutation at the Sl locus (Sl/Sl^{d}), which encodes for SCF, are not only mast cell deficient but exhibit severe anaemia (McCulloch, Siminovich, Till, Russel & Bernstein, 1965). Therefore a number of haematological parameters were measured to determine the effects of anti-SCF treatment on haemopoiesis.

Red blood cell count, haematocrit and haemoglobin concentration were unaltered in rats treated for 4 days with either anti-SCF or normal sheep IgG antibodies when compared with pre-treatment values. The white cell count of the anti-SCF-treated group was depressed significantly when compared with normal IgG-treated control rats ($p < 0.02$; Table 15). Differential cell counts revealed that while the proportion of circulating neutrophils was significantly depressed in both anti-SCF and normal IgG-treated groups ($p < 0.001$ and $p < 0.005$ respectively; Table 15) the proportions of lymphocytes, monocytes and eosinophils were unchanged.

Table 15 Haematological parameters of rats treated for 4 days with sheep anti-SCF (anti-SCF) or with normal sheep IgG (control) compared with pre-treatment (day 0) controls.

	Day	n	WBC ($x10^{10}$/L)	RBC ($x10^{12}$/L)	Hb (g/dL)	Hct (%)	Differential count (%)			
							Neutrophil	Lymphocyte	Monocyte	Eosinophil
Control	0	10	1.1 ± 0.05	6.7 ± 0.1	15.4 ± 0.3	40.5 ± 0.5	14.9 ± 1.54	78 ± 2.0	6.1 ± 0.7	1.1 ± 0.4
anti-SCF	5	5	0.6 ± 0.04*	6.3 ± 0.2	14.6 ± 0.7	39.4 ± 1.1	5.0 ± 1.1	85 ± 2.5	7.8 ± 1.9	1.8 ± 0.7
Control	5	5	1.1 ± 0.16	6.1 ± 0.4	14.9 ± 0.8	38.8 ± 1.6	5.2 ± 1.56	88 ± 1.8	5.0 ± 1.5	1.8 ± 0.7

* = $p < 0.02$ by two-tailed Student's t-test vs. day 5 control. WBC, white blood cell; RBC, red blood cell; Hb, haemoglobin; Hct, haematocrit.

6.4 Discussion

Treatment of normal rats with anti-SCF for 4 days significantly depleted mast cells from the peritoneal cavity and also depleted RMCP I from other tissues. Perhaps more surprisingly this treatment also totally ablated the intestinal MMC population and virtually eliminated circulating RMCP II. An additional effect of anti-SCF treatment is that it diminished the numbers of all circulating white blood cells, probably because of an effect on haematopoietic progenitor cells (Williams *et al* 1991).

One interpretation of these results is that endogenous SCF is necessary for the survival of mature mast cell populations. rrSCF can maintain mouse mast cell survival *in vitro* (Mekori, Oh & Metcalfe, 1993; Iemura *et al* 1994) or *in vivo* (Iemura *et al* 1994) by suppressing apoptosis, and the abrogation of this effect by anti-SCF could account for the current findings. Of course, rrSCF^{164} can promote the recruitment of mast cell precursors *in vivo* (Tsai *et al* 1991b; Zsebo, Williams, Geissler, Broudy, Martin, Atkins, Birkett, Okino & Murdock, 1990) and can favour their development into mature mast cells (Tsai *et al* 1991b; Zsebo *et al* 1990). It can also synergise with IL-3 to promote rat mast cell proliferation (Haig *et al* 1994; Tei *et al* 1994). These additional effects of SCF may also have been antagonised by anti-SCF treatment.

However, the total ablation of mature MMCs from the intestinal mucosa of normal rats by anti-SCF cannot easily be explained by a suppression of recruitment of mast cell precursors alone. The half-life of mucosal mast cells in rat intestine has been estimated at around 40 days (Enerbäck & Norrby 1989) and blocking recruitment for 4 days would be expected to have a negligible effect on mast cell numbers. These

findings can perhaps best be explained by effects of anti-SCF on the SCF-dependent suppression of mast cell apoptosis. *In vitro* murine BMMC, maintained in SCF, begin to undergo apoptosis within hours of the removal of SCF from the culture medium (Iemura *et al* 1994). It is interesting to note that whilst mucosal mast cells are totally ablated by anti-SCF treatment the connective tissue-type mast cells of the peritoneal cavity, and probably other tissue locations, are only partially depleted. Similarly, mucosal mast cells in rat jejunum were significantly depleted within hours of treatment with the corticosteroid dexamethasone (Soda, Kawabori, Perdue & Bienenstock, 1991); a treatment which suppresses cytokine expression (Arya, Wong-Staal & Gallo 1984, Culpepper & Lee 1985). Corticosteroid treatment, on the other hand, has a negligible effect on rat PMC numbers (King, Miller, Newlands & Woodbury, 1985). This evidence, taken together, shows that MMC are not only highly dependent on the T cell-derived cytokines IL-3 and IL-4 but that they also require SCF for their survival *in vivo*. On the other hand connective tissue-type mast cells are not only IL-3 and IL-4 independent but also have much less of a dependency on SCF for their immediate survival.

7. STEM CELL FACTOR IN MAST CELL HYPERPLASIA ASSOCIATED WITH INTESTINAL NEMATODIASIS

7.1 Introduction

In murine rodents, infection with intestinal nematodes induces a striking hyperplasia of intestinal MMCs, which is accompanied by increases in the levels of MMC-associated neutral serine proteases in intestinal tissues and increases in the concentrations of these proteases in the blood (Miller *et al* 1983; Woodbury *et al* 1984, Huntley *et al* 1990b). SCF and T cell-dependent mechanisms both contribute to the intestinal MMC hyperplasia associated with nematode infection. In comparison with normal mice or rats, those with mutations that markedly diminish *c-kit* receptor tyrosine kinase activity, such as W/W^v mice (Reed 1989) or W^s/W^s rats (Arizono *et al* 1993), exhibit no or greatly diminished hyperplasia of intestinal MMCs in response to infection with *N. brasiliensis* (Reed 1989; Arizono *et al* 1993) or *T. spiralis* (reviewed in Reed 1989). Both intestinal MMC hyperplasia and the spontaneous expulsion of the parasites are diminished in *T. spiralis*-infected mice that have been treated with an antibody to the *c-kit* receptor (Grencis *et al* 1993). Moreover, treatment of normal rats with *E. coli*-derived rrSCF^{164}, which represents virtually the entire extracellular ligand domain of SCF and which possesses high biological activity (reviewed in Galli *et al* 1994), induces significant hyperplasia of gastrointestinal MMCs as well as connective tissue-type mast cells (Tsai *et al* 1991a). On the other hand, athymic 'nude' mice fail to develop MMC hyperplasia in response to nematode infection, whereas this response is restored in nude animals treated by adoptive

transfer of T cells (reviewed in Reed 1989). Moreover, in normal mice, treatment with either anti-IL-3 or anti-IL-4 antibodies, or, even more effectively, treatment with both antibodies, significantly suppresses intestinal mast cell hyperplasia in response to *N. brasiliensis* infection (Madden *et al* 1991). *In vitro* evidence indicates that SCF and IL-3 can have synergistic effects in promoting the proliferation of rat mast cells with phenotypic similarities to MMCs (Haig *et al* 1994; Tei *et al* 1994). In addition, during *N. brasiliensis* infection, PMC are induced to begin expressing the MMC protease RMCP II (Huntley *et al* 1993). This is in accord with the results reported in chapter 5, where *in vivo* treatment of rats with SCF and/or IL-3 also induces PMC to express RMCP II as well as RMCP I.

Taken together, this evidence indicates that the cytokine-dependent regulation of MMC hyperplasia during nematode infection in murine rodents, while complex, importantly involves both SCF and T cell-derived cytokines such as IL-3 and IL-4.

7.2 Experimental Design

In this chapter the role of SCF in both the mucosal mast cell hyperplasia associated with nematode infection and the induction of RMCP II expression in PMC were investigated by treatment of *N. brasiliensis* or *T. spiralis*-infected rats with a polyclonal sheep antibody to rat SCF. The objective of these experiments was to determine whether this treatment would alter mast cell numbers or protease expression in small intestine or peritoneal cavity. In addition, the effects of anti-SCF treatment on parasite fecundity were assessed in *N. brasiliensis*-infected rats by monitoring faecal egg-counts. This data was compared with faecal egg-counts from the rats treated with $rrSCF^{164}$ by daily intravenous injection as described in chapter 5.

Rats were inoculated intraperitoneally with 1 mg sheep anti-rrSCF[164] or normal sheep IgG. Because of the tissue and pulmonary migration of the L_3 and L_4 larval stages of *N. brasiliensis*, rats infected with this parasite were treated on day 3 of infection when the L_4 larvae first reached the intestine and on days 5, 7, 10 and 12 thereafter. For rats infected with *T. spiralis*, treatment commenced on day 0 and continued on days 3, 5, 7 and 10.

In the experiment where the rats were infected with *N. brasiliensis*, groups of rats were killed on days 6, 10 and 14 after infection and where infection was with *T. spiralis* groups were killed on days 6, 10 and 12. In both experiments the rats were killed by exsanguination under deep anaesthesia followed by cervical dislocation.

Samples of small intestine were collected and fixed in paraformaldehyde for histological examination (2.2.8) or stored at -20°C for subsequent measurement of RMCP I and II by ELISA (2.2.9, 2.2.10). Samples were also collected by peritoneal lavage for evaluation of peritoneal mast cell numbers, phenotype assessment by immunocytochemistry and assay of RMCP I and II by ELISA. In a separate experiment, rats infected with *N. brasiliensis* were treated with anti-SCF antibodies on days 10 to 13 inclusive, of infection to determine whether an established mast cell hyperplasia was dependent on SCF. Blood samples were taken, to determine haematological parameters (2.2.7), at the start of the experiment, on day 0, and again on day 10 at the commencement of anti-SCF treatment. The animals were killed on day 14 and blood samples were again collected for haematology and for the measurement of RMCP II by ELISA. In addition, samples of small intestine were collected for histology (2.2.8) and ELISA (2.2.9, 2.2.10) on day 14.

7.3 Results

7.3.1 Anti-SCF treatment: effects of treatment on the mucosal mast cells of the small intestine.

Since anti-SCF treatment down-regulated the number of peritoneal mast cells in normal rats (chapter 6), it was important to determine whether anti-SCF treatment in association with *N. brasiliensis* or *T. spiralis* infection had a similar, or different effect on MMC.

7.3.1.1 Infection with *N. brasiliensis*.

N. brasiliensis-infected rats were first treated with anti-SCF or with normal sheep IgG on day 3 of infection, when the worms begin to emerge into the gastrointestinal tract. As expected, mast cell counts in both control IgG and specific antibody-treated groups were low on day 6 (Figure 16a). This result is in accord with previously published data that *N. brasiliensis* infection produces an initial reduction in mast cell densities in the intestines (Miller & Jarrett 1971) and other, distant, sites (Huntley *et al* 1993).

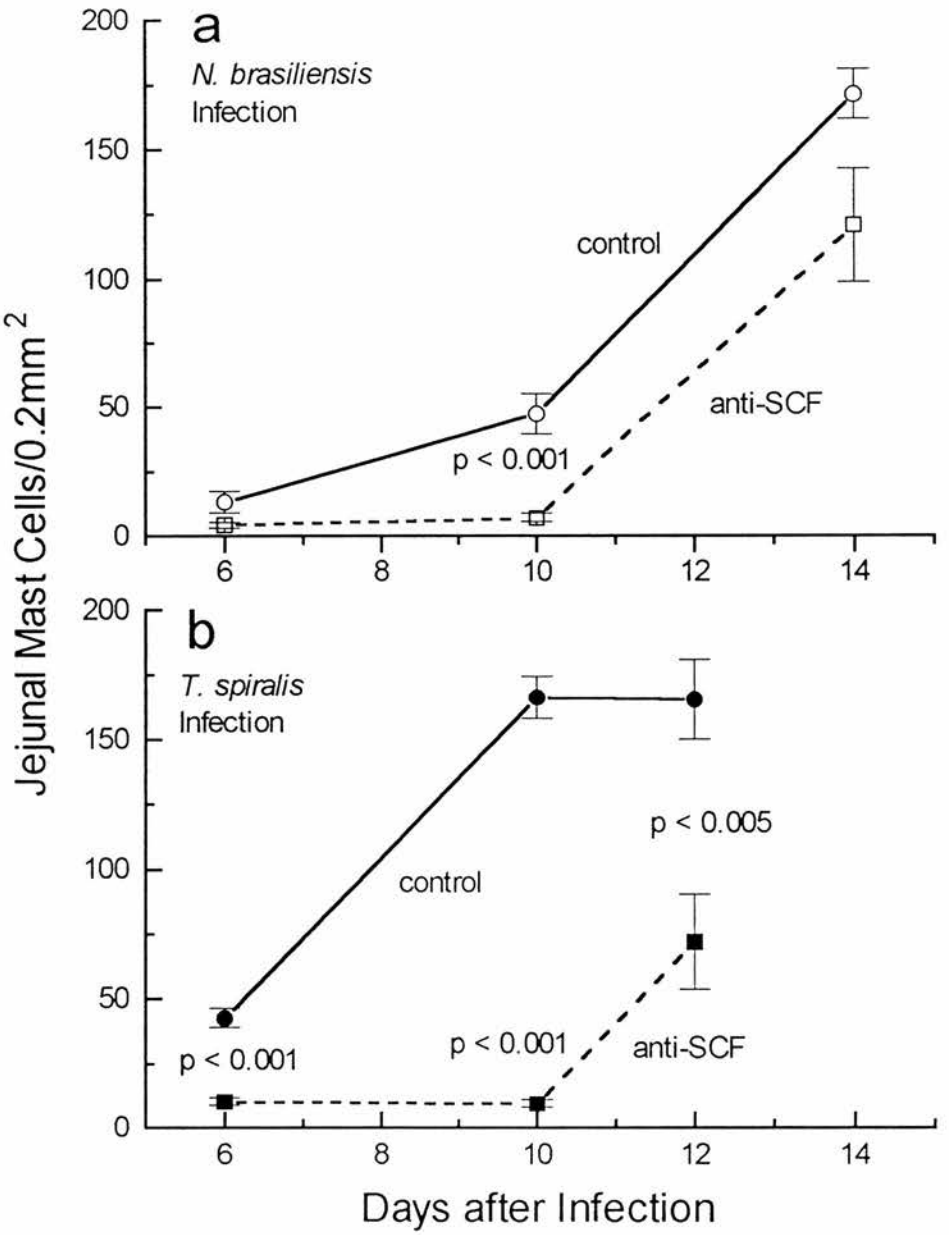


Figure 16. Mast cell densities in jejunum of rats infected with *N. brasiliensis* or *T. spiralis* and treated with anti-SCF antibodies.

a) *N. brasiliensis*-infected rats treated with anti-rrSCF^{164} (❑) or normal sheep IgG (○), or b) *T. spiralis*-infected rats treated with anti-rrSCF^{164} (■) or normal sheep IgG (●). Mast cell densities in anti-SCF treated rats were compared with the equivalent time points in control rats by Student's two sample t-test.

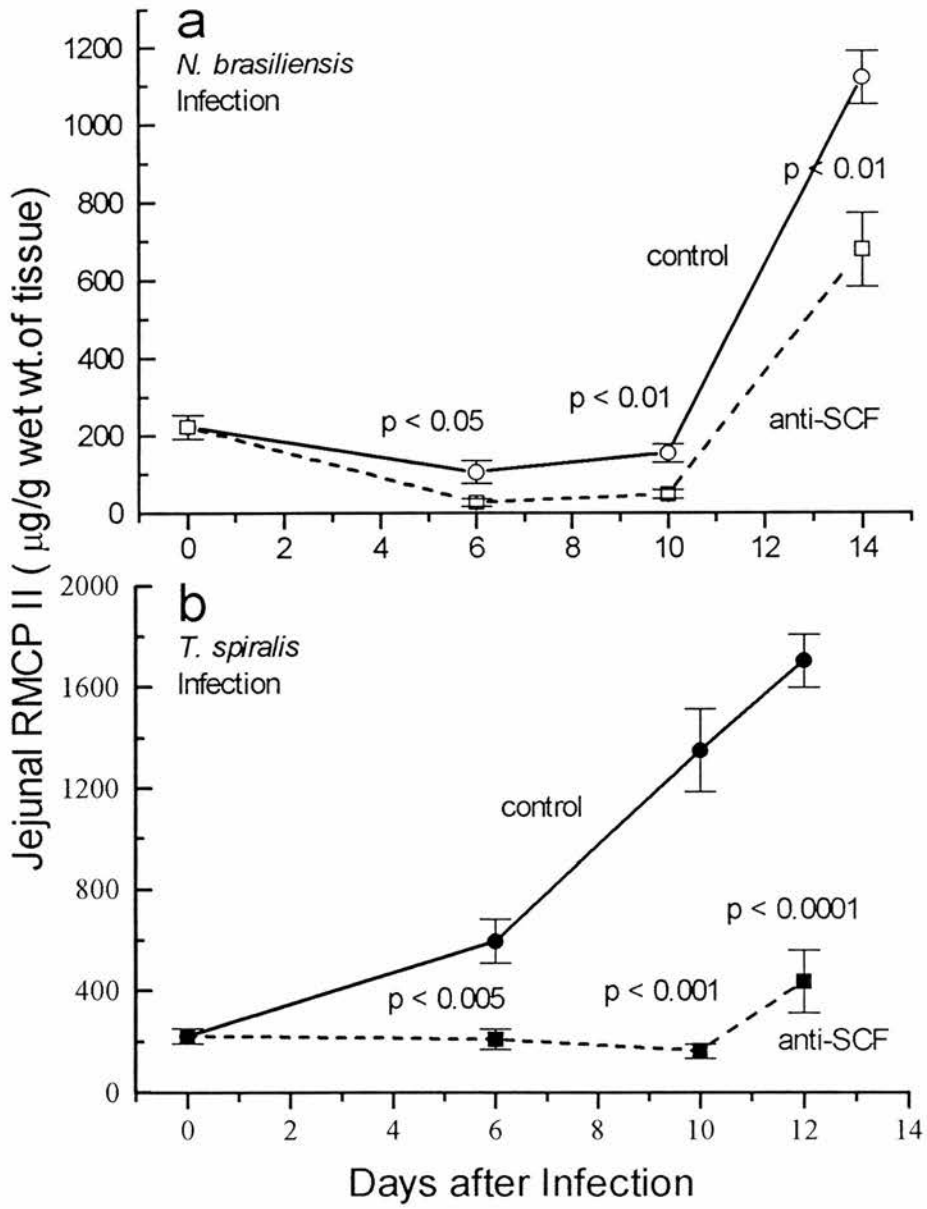


Figure 17. RMCP II content of jejunum of rats infected with *N. brasiliensis* or *T. spiralis* and treated with anti-SCF antibodies.

a) ***N. brasiliensis*** **-infected rats treated with anti-rrSCF^{164} (❑) or normal sheep IgG (○), or b) *T. spiralis* -infected rats treated with anti-rrSCF^{164} (■) or normal sheep IgG (●). Jejunal RMCP II concentrations in anti-SCF treated rats were compared with the equivalent time points in control rats by Student's two sample t-test.**

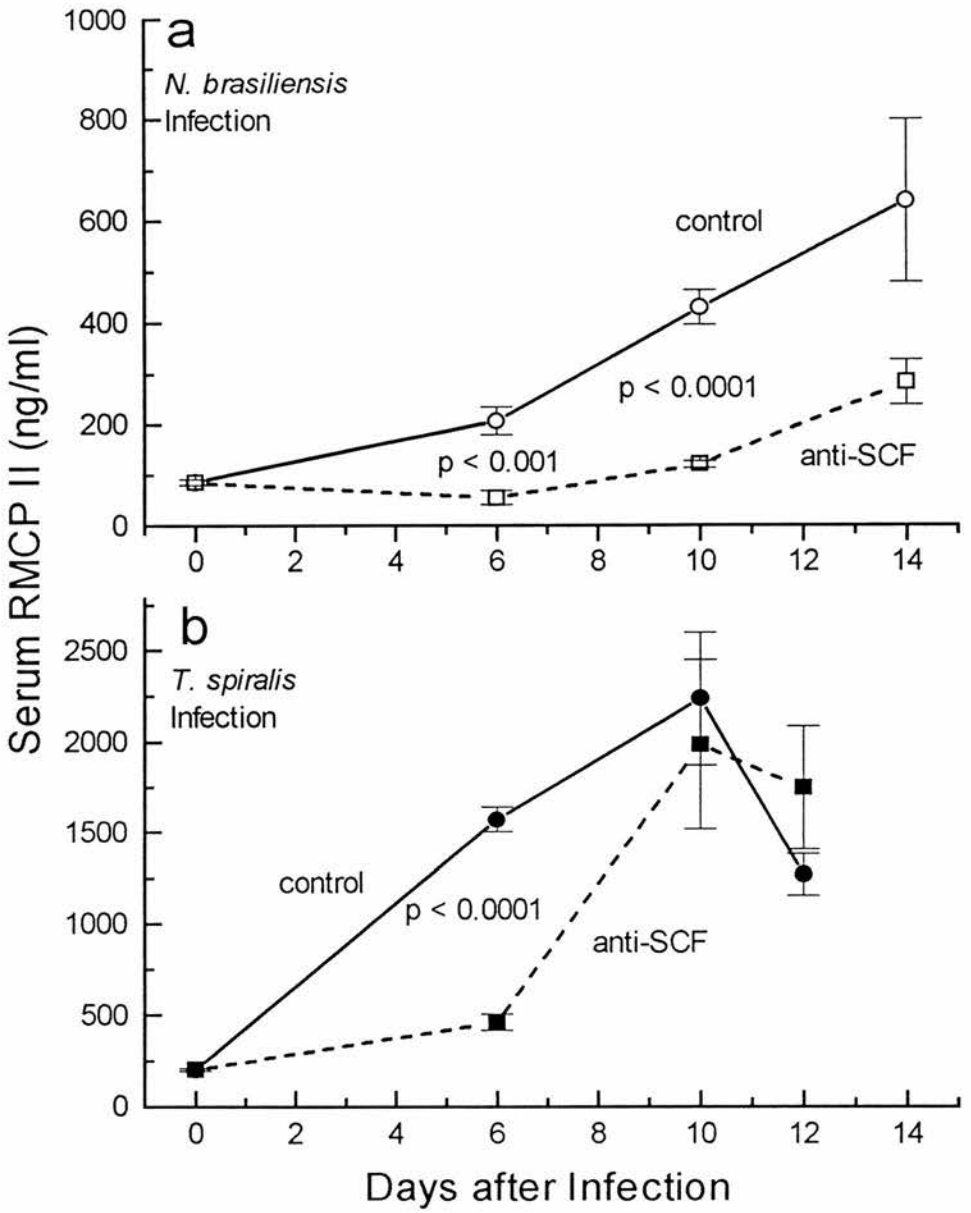


Figure 18. Serum concentrations of RMCP II in rats infected with *N. brasiliensis* or *T. spiralis* and treated with anti-SCF antibodies.
a) ***N. brasiliensis*-infected rats treated with anti-rrSCF^{164} (❑) or normal sheep IgG (○), or b) *T. spiralis*-infected rats treated with anti-rrSCF^{164} (■) or normal sheep IgG (●). Serum RMCP II concentrations in anti-SCF treated rats were compared with the equivalent time points in control rats by Student's two sample t-test.**

However anti-SCF treatment resulted in significant inhibition of mast cell hyperplasia (Figure 16a). This effect was most notable on day 10 of infection ($p < 0.0001$ vs. the corresponding value in control rats) but was observed throughout the entire time course of the infection ($p < 0.0005$ vs. control values by ANOVA). The anti-SCF-treated rats also had significantly reduced levels of RMCP II in the jejunal mucosa at all time points tested (Figure 17a) as well as over the entire time course of the infection ($p < 0.0005$ vs. control values by ANOVA). This reduced mucosal concentration of RMCP II was paralleled by a highly significant depression of RMCP II concentrations in blood 6 and 10 days after infection ($p < 0.001$ at either interval, $p < 0.0005$ over the entire time course of the infection; Figure 18a). Simple regression analysis of the mast cell densities and RMCP II concentrations in the jejunum of all *N. brasiliensis*-infected animals gave a highly significant correlation ($R = 0.96$, $p < 0.0001$, Figure 19a).

To determine whether SCF remains a significant factor in MMC hyperplasia or survival even after the onset of the intestinal MMC response, groups of rats were treated with anti-SCF or normal sheep IgG daily from day 10 of infection, when mast cell hyperplasia was well under way (e.g. see Figure 16a). The rats treated with anti-SCF showed a 32% depletion of RMCP II from the small intestine on day 14 compared with the values for normal sheep IgG-treated controls ($p < 0.01$; Table 16), with a concomitant 32% decrease in mast cell density ($p < 0.03$; Table 16). The 15% decrease in the serum RMCP II concentrations in the anti-SCF-treated rats was not statistically significant (Table 16) nor was there any significant alteration in the haematological parameters measured, as a result of the anti-SCF treatment (Table 17).

Table 16 Jejunal mast cell densities and RMCP II concentrations in intestine and serum in rats treated with anti-SCF or normal sheep IgG from day 10 of *N. brasiliensis* infection.

	Day 0	Day 10	day 14 control	day 14 anti-SCF
jejunal mast cells (cells/0.2 mm^2)	ND	ND	65 ± 7	44 ± 3*
Jejunal RMCP II (μg/g wet wt.)	ND	ND	1752 ± 106	1186 ± 111**
serum RMCP II (ng/ml)	95 ± 4	360 ± 21	453 ± 62	386 ± 121

Comparison of day 14 anti-SCF treatment group data with day 14 control data by two-sample Student's t-test. *$p < 0.03$, **$p < 0.01$ vs value, ND = not determined.

Table 17 Haematological parameters in rats treated with anti-SCF or normal sheep IgG (control) from day 10 of N. brasiliensis infection.

	n	WBC ($x10^{10}$/L)	RBC ($x10^{12}$/L)	Hb(g/dL)	Hct %
day 0	10	1.37 ± 0.05	6.7 ± 0.08	15.7 ± 0.2	40.0 ± 0.7
day 10	10	1.11 ± 0.1	7.1 ± 0.2	16.1 ± 0.3	39.2 ± 1.2
day 14 control	5	1.2 ± 0.05	6.7 ± 0.1	15.1 ± 0.3	40.4 ± 0.8
day 14 anti-SCF	5	1.1 ± 0.1	6.4 ± 0.1	14.5 ± 1.6	37.4 ± 4.2

The day 0 and day 10 values presented are pooled data from both control and anti-SCF treatment groups before treatment with antibodies commenced. WBC, white blood cell; RBC, red blood cell; Hb, haemoglobin; Hct, haematocrit.

7.3.1.2 Infection with *T. spiralis.*

Infective *T. spiralis* larvae establish themselves in the intestine within hours of oral challenge, therefore treatment with anti-SCF was first given at the time of infection, on day 0. Mast cell hyperplasia occurs sooner during trichinosis than in *N. brasiliensis*-infected rats, and anti-SCF treatment significantly depressed mast cell densities on days 6, 10 and 12 of infection ($p < 0.001$, $p < 0.001$ and $p < 0.005$ respectively; $p < 0.0005$, by ANOVA over the entire course of the infection; Figure 17b) when compared with values in normal IgG-treated controls. Again this result was paralleled by values for mucosal concentrations of RMCP II, which were significantly reduced 6, 10 and 12 days after infection ($p < 0.005$, $p < 0.001$ and $p <$

0.001 respectively; $p < 0.0005$, by ANOVA over the entire course of infection; Figure 17b) in anti-SCF treated rats. The relationship between mucosal RMCP II and mast cell densities, when analysed by simple regression analysis, showed a positive correlation ($R = 0.90$, $p < 0.0001$, Figure 19b). In contrast with responses in *Nippostrongylus*-infected rats, the systemic secretion of RMCP II was depressed on day 6 ($p < 0.001$) but not at other time points (Figure 18b). As previously reported (Woodbury *et al* 1984) it was found that *T. spiralis*, when compared with *N. brasiliensis* infection, was associated with a greater, and earlier, systemic release of RMCP II (compare Figure 18a and b).

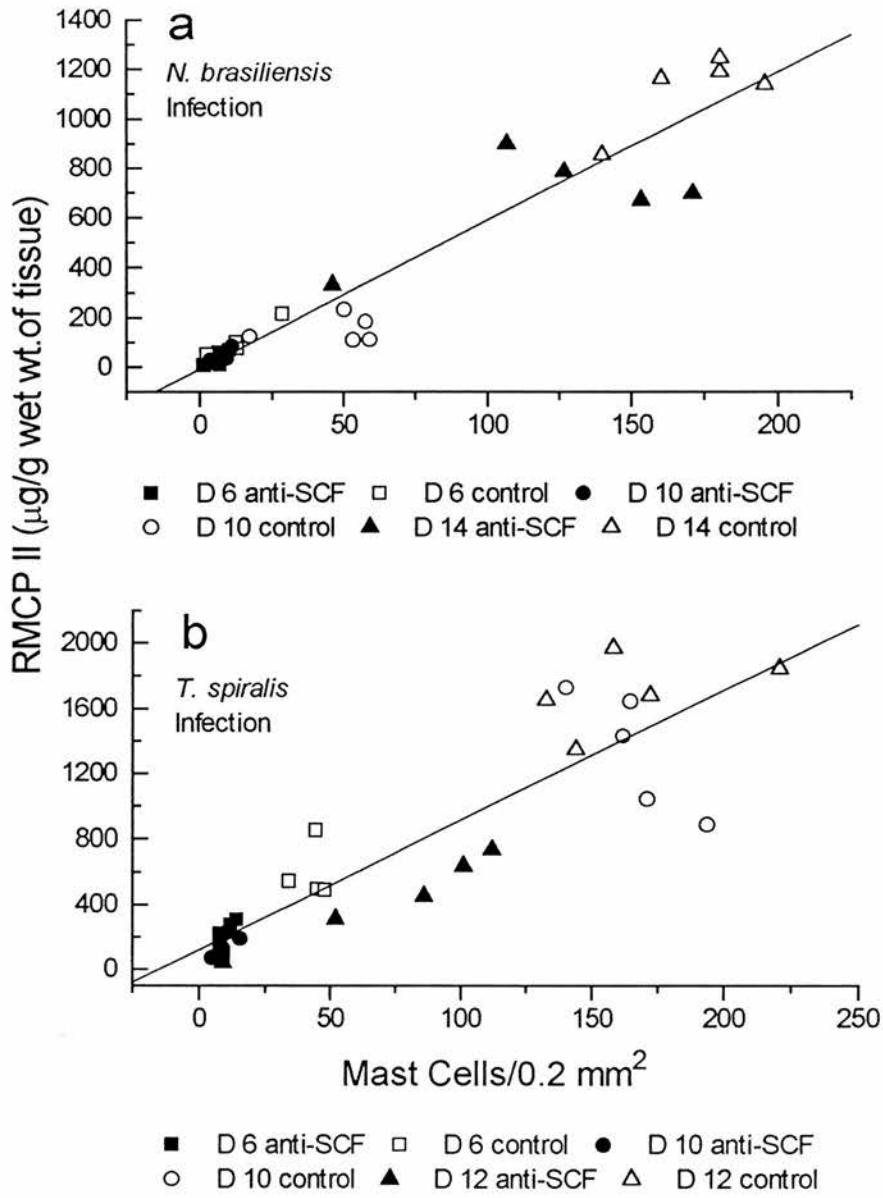


Figure 19. Correlation between mast cell densities and RMCP II concentrations in small intestine of parasitised rats, treated with anti-SCF antibodies.

Plot a) *N. brasiliensis*-infected rats. Plot b) *T. spiralis*-infected rats. The control data are represented by open symbols and the anti-SCF treatment data by closed symbols.

7.3.2 Anti-stem cell factor treatment: effects on peritoneal mast cell populations in parasitised rats.

N. brasiliensis-infected rats, treated with anti-SCF, showed a significant decrease ($p < 0.003$ by ANOVA) in PMC numbers compared with controls but the amounts of RMCP I and II per mast cell was not significantly altered. Similarly, in *T. spiralis*-infected rats, there was also a significant depletion in PMC numbers at days 10 and 12 of infection. This was accompanied by an apparent increase in the RMCP I content of the mast cells. However, due to the low numbers of mast cells detected, this may be due to sampling error and is probably not biologically important. These data are reported fully in Appendix 1, section 12.1.

7.3.3 Development of an antibody response to sheep immunoglobulins in rats treated with the polyclonal sheep anti-SCF antibodies.

Because treatment of rats was with a polyclonal sheep antibody, it was possible that antibodies to sheep IgG would be generated during treatment. This was confirmed when, on day 6 of treatment with either sheep anti-SCF or normal sheep IgG, *T. spiralis*-infected rats had a sheep IgG-specific antibody titre of 320, as assessed by ELISA, when compared with pre-treatment sera from the same group of rats ($p < 0.05$). Interestingly, although the assay of sera from day 12 of treatment gave somewhat higher absorbances than that from the day 6 groups the titre remained at 320 ($p < 0.05$). To determine whether this was a genuine anti-sheep IgG response or simply a reflection of up-regulated IgG production as a result of infection, the sera from *N. brasiliensis*-infected rats which had not been treated with sheep immunoglobulins were also assayed. No significant difference in absorbance was

found at day 3, 6, 9 or 12 of infection when compared with serum samples obtained from the same group of rats prior to infection. The protocol for determining anti-sheep antibody titre is described in Materials and Methods (2.2.11).

7.3.4 Effect of rrSCF^{164} or anti-SCF on parasite fecundity.

The effect of the administration of anti-SCF on the fecundity of *N. brasiliensis* was determined by monitoring egg output in the faeces of the rats. In the experiments in which rats were treated with anti-SCF or normal sheep IgG, the worm egg-output was monitored over the latter part of the infection to determine whether depressing the intestinal mast cell response would lead to an alteration of worm fecundity. Animals which were treated with anti-SCF (and had reduced densities of jejunal MMC) had significantly lower faecal egg-counts on both days 8 and 9 than did the normal IgG-treated controls ($p < 0.03$ at both time intervals; Figure 20). Over the entire time course of the response shown in Figure 20, values for anti-SCF treated rats were significantly lower than those in control rats by ANOVA ($p < 0.001$). However egg output had virtually ceased by day 11 in both groups.

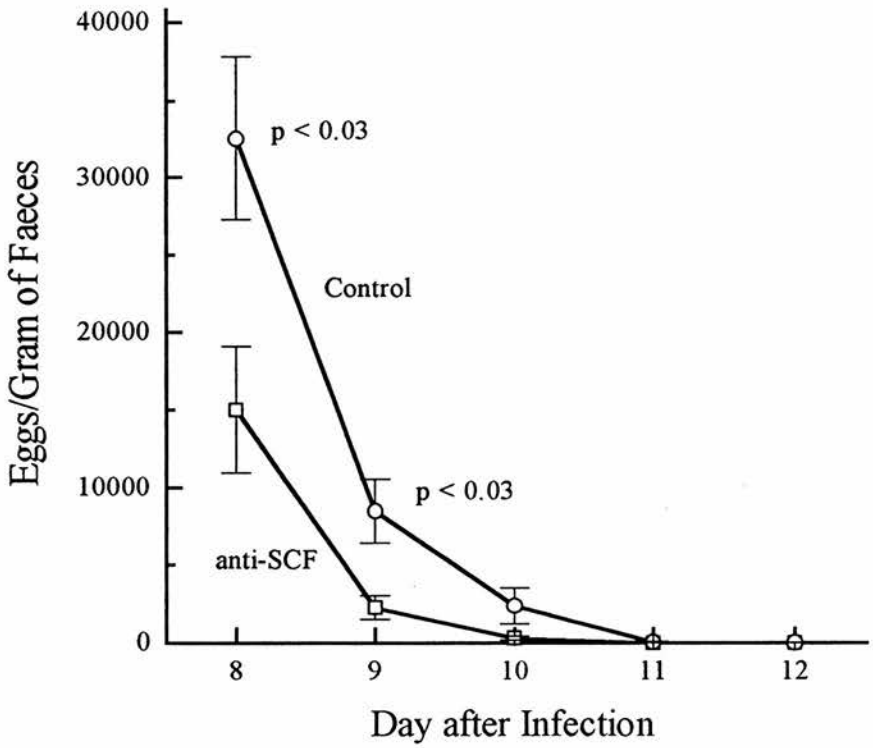


Figure 20 Faecal egg-output data from *N. brasiliensis*-infected rats treated with sheep anti-SCF.
Anti-SCF-treated (❑) or normal sheep IgG-treated, control rats (○).

7.4 Discussion

These findings provide additional evidence that SCF importantly contributes to the MMC hyperplasia that occurs during nematode infection in rats, show that this response can be markedly suppressed by administration of an anti-SCF antibody, and raise interesting questions about the role of SCF and mast cells in the regulation of parasite fecundity during *N. brasiliensis* infection.

To assess the importance of endogenous SCF in the intestinal MMC response to nematode infection in normal rats a polyclonal sheep anti-rat SCF antibody was administered to rats infected with *N. brasiliensis* or *T. spiralis*. Treatment with anti-SCF beginning at the time of infection profoundly interfered with mast cell hyperplasia in the jejunum and significantly diminished the systemic release of RMCP II after infection with either *N. brasiliensis* or *T. spiralis*. These effects were most marked at the earlier intervals of infection, probably because of the later development of antibodies to sheep IgGs in the anti-SCF-treated rats. When anti-SCF was administered to rats that had already undergone expansion of jejunal MMCs in response to *N. brasiliensis* infection, anti-SCF treatment resulted in a significant decrease in both intestinal RMCP II concentrations and mast cell numbers, but produced neither a significant depression of RMCP II concentrations in the serum nor a significant change in numbers of peripheral blood white cells.

One interpretation of these findings with anti-SCF is that endogenous SCF is necessary for the survival of mast cell populations, including both baseline populations of MMCs in uninfected rats (Chapter 6) and the markedly expanded MMC

populations in nematode-infected rats. rrSCF^{164} can maintain mouse mast cell survival *in vitro* (Mekori *et al* 1993; Iemura *et al* 1994) or *in vivo* (Iemura *et al* 1994) by suppressing apoptosis, and the abrogation of this effect by anti-SCF could account for the current results. Of course, rrSCF^{164} can promote the recruitment of mast cell precursors *in vivo* (Tsai *et al* 1991b; Zsebo *et al* 1990) and can favour their development into mature mast cells (Tsai *et al* 1991b; Zsebo *et al* 1990). It can also synergise with IL-3 to promote rat mast cell proliferation (Haig *et al* 1994, Tei *et al* 1994) probably through up regulation of IL-3 receptors (Liu, Cutler, Mui & Krystal, 1994). These additional effects of SCF also may have been antagonised by anti-SCF treatment, particularly when treatment was begun at the time of infection.

Rats, infected with *N. brasiliensis* or *T. spiralis* and treated with anti-SCF all showed marked depletion of peritoneal mast cells and, in the *T. spiralis*-infected rats, there is an apparent increase in the RMCP I content of the PMC, although is most probably due to sampling error due to the low numbers of PMC recovered from this group. The up-regulation of RMCP II expression, so marked in the earlier experiments (Table 7, Table 8; Chapter 4), was less pronounced, but still significant, in both *N. brasiliensis* and *T. spiralis* infections where the rats were treated with control immunoglobulins (Table 19, Table 20). RMCP II production by serosal mast cells was significantly reduced, in both infection models, by treatment with anti-SCF (Table 19, Table 20). This clearly indicates that SCF is a necessary component in the up-regulation of RMCP II expression by serosal mast cells.

In *T. spiralis* infection of mice, treatment with an antibody directed against *c-kit*, the SCF receptor, not only diminished intestinal mast cell hyperplasia but also

reduced parasite expulsion (Grencis *et al* 1993). This finding supports the widely held view that mast cells can represent an important component of host immunity to parasite infection. Yet we found that treatment with rrSCF^{164}, which accelerated the development of the MMC response (chapter 5), did not diminish and actually somewhat increased parasite fecundity during *N. brasiliensis* infection. This effect was particularly evident at day 8 of infection, the day on which rrSCF^{164}-treated rats had significantly more jejunal MMCs than did the control rats (chapter 5, Table 9). Moreover, treatment with anti-SCF, which markedly suppressed the MMC hyperplasia associated with *N. brasiliensis* infection, substantially (by > 50%) and significantly ($p = 0.001$) diminished *N. brasiliensis* egg production during the later stages of infection.

In considering these findings, it should be noted that genetically mast cell deficient (*W/W^v, Sl/Sl^d)* mice or *W^s/W^s* rats exhibit little or no impairment in their ability to expel a primary infection with *N. brasiliensis* (reviewed in Reed 1989; Arizono *et al* 1993), findings which suggest that mast cells are not essential host-protective cells against primary infection with this nematode. The current findings in rats with a normal genotype raise the interesting possibility that mast cells (and /or some other SCF-responsive cell type) may actually have some effects which favour parasite fecundity during *N. brasiliensis* infection. Perhaps increased numbers of intestinal MMCs, and the activation of these cells for mediator release, which can result in increased permeability of gut blood vessels and intestinal mucosa (Scudamore *et al* 1995), promote the nutrition of the parasites. The mechanisms that might account for the apparent decreased fecundity of *N. brasiliensis* in rats treated with

anti-SCF are the subject of further studies. However, it has been shown recently that suppression of IgE levels can decrease both the worm burden and egg production associated with primary infection with *Schistosoma mansoni* in mice (Amiri, Haak-Frendscho, Robbins, McKerrow, Stewart & Jardieu, 1994). Taken together with the current results, these findings suggest that, in certain parasite infections, aspects of the mast cell-and IgE-dependent immune responses that are elicited by the organisms may have effects which are more advantageous to the parasite than to the host.

8. STEM CELL FACTOR DEPENDENT HYPERPLASIA OF MUCOSAL-TYPE MAST CELLS BUT NOT EOSINOPHILS IN *SCHISTOSOMA MANSONI*-INFECTED RATS.

8.1 Introduction

The cellular immune response to many helminth parasites is characterised by hyperplasia of mast cells and eosinophils both locally, at the site of infection and systemically. Both of these cell types derive from haemopoietic progenitor lineages in bone marrow which differentiate and proliferate under the influence of an array of cytokines and growth factors. The mechanisms regulating mast cells through T cell-derived cytokines and SCF have been described in detail previously (chapter 5).

IL-3, IL-5 and GM-CSF all have a role in controlling proliferation and differentiation of eosinophil populations (reviewed by Sanderson, 1991). Additionally, both IL-3 and IL-5 can act in synergy with SCF *in vitro* to give enhanced growth of eosinophils from an eosinophil precursor cell line (Kobayashi 1993). It is clear from this evidence, therefore that SCF has an important role to play in generating the cellular immune response to parasites. Both mast cell and eosinophil numbers increase substantially in the rat in response to infection with the intra-vascular parasite *Schistosoma mansoni*. Mast cell numbers are elevated in both intestinal mucosa and liver (Miller, Newlands, McKellar, Inglis, Coulson & Wilson, 1994) and there is a significant increase in the numbers of circulating eosinophils (Phillips, Bentley, Linette, Doughty & Capron, 1983). Rat eosinophils, which mediate worm damage *in vitro* (Capron, Bazin, Joseph & Capron, 1981), may have a direct anti-parasite role. Furthermore, mast cells, which are functionally active during the response to *S.*

mansoni, as can be seen from the systemic release of the mucosal mast cell-associated protease RMCP II (Miller *et al* 1994), may have an accessory role mediated via anaphylactic release of a range of immune and inflammatory mediators through membrane bound, parasite-specific, IgE (Capron, Dessaint, Capron, Joseph & Pestel, 1980). In this chapter the role of SCF in mast cell and eosinophil hyperplasia was examined by treating *S. mansoni*-infected rats with a polyclonal sheep anti-rat SCF antibody to determine whether SCF depletion altered mast cell or eosinophil numbers or concentrations of RMCP II in liver or small intestine.

8.2 Experimental Design

Fischer F344 inbred female rats, were infected with approx. 2500 *S. mansoni* cercariae via shaved abdominal skin by the ring technique (Smithers & Terry 1965). Blood samples were taken on days 18, 21, 24, 27, and 30 after infection by tail-snip (2.2.7). Serum was harvested and stored at -20°C. The rats were treated with 1 mg sheep anti-SCF or normal sheep IgG on days 21, 24, 27 and 30 by intraperitoneal injection. These procedures were carried out at the University of York by Prof. R.A. Wilson and Dr P. Coulson.

On day 32 the rats were anaesthetised and blood samples taken as before. The hepatic portal system was perfused to recover adult worms and samples of liver and small intestine were collected for histology (2.3.5) and measurement of mast cell proteases by ELISA. Rat mast cell proteases I and II were measured in serum and tissue extracts by antibody capture ELISA (2.2.9, 2.2.10). Data are presented as mean

± sem and were analysed by Student's two sample t-test or, for time course data by, analysis of variance (ANOVA).

8.3 Results and Discussion

Serum concentrations of RMCP II increased in control rats from around 400 ng/ml on day 18 of infection with *S. mansoni* to 1045 ng/ml by day 32. (Figure 21); this is in good agreement with previously reported results (Miller *et al* 1994). In test rats, by day 24 of infection with *S. mansoni*, three days after the first injection of anti-SCF antibody, the concentrations of RMCP II in serum were significantly depressed ($p < 0.0002$) when compared with values in the control rats treated with normal sheep IgG. The RMCP II concentrations in the blood of anti-SCF treated rats decreased until only 12 ng/ml were detected on day 32 of infection, over 30-fold lower than on day 21 and 87-fold lower when compared to normal sheep IgG-treated controls (Figure 21; Newlands, Coulson & Wilson, 1995b). Comparison of the time course data for serum RMCP II concentrations in anti-SCF-treated and control rats showed a highly significant difference ($p < 0.0005$ by ANOVA) between the two treatments. Systemic concentrations of RMCP II in anti-SCF-treated and control rats were reflected by both the tissue protease concentrations, and mast cell counts, in liver and jejunum on day 32 of infection (summarised in Table 18; Newlands *et al* (1995b); these values were both reduced by > 95% in each tissue. RMCP I, the connective tissue mast cell-associated protease, was not detectable in the jejunum of anti-SCF treated rats whilst there was a > 8-fold lower level ($p < 0.001$) in liver. Similarly concentrations of RMCP II in the tissues showed > 25-fold lower values compared with controls (Table 18). This was in sharp contrast to the numbers of

eosinophils in both liver and small intestine, which were not significantly altered by anti-SCF treatment when compared with control animals (Table 18). Fewer worms were recovered by perfusion of the liver of anti-SCF-treated rats (159 ± 10.7) than from normal sheep IgG-treated (control) rats (189 ± 15.2) although the difference did not achieve statistical significance. This slight decrease in parasite numbers is in keeping with the previous observation that *N. brasiliensis* fecundity was reduced in anti-SCF treated rats (Chapter 7), and also that suppression of the IgE response with anti-IgE antibodies decreased both the worm burden and egg production associated with *S. mansoni* infection in mice (Amiri *et al* 1994). These data suggest that, in at least some parasitic infections, worm survival and fecundity may be enhanced by some aspect of the mast cell-IgE dependent immune responses.

Unaltered eosinophil numbers in the liver and jejunum of test and control rats suggest that eosinophil recruitment in parasitic infections is not significantly dependent on the presence of SCF or of mast cells, although SCF does synergise with IL-3 or IL-5 in promoting growth in an eosinophil cell line *in vitro* (Kobayashi, 1993). Similarly, when mice infected with *N. brasiliensis* were treated with antibody to IL-3, which suppressed mast cell hyperplasia, the associated eosinophilia in peripheral blood was undiminished (Madden *et al* 1991) despite the fact that IL-3 is a recognised eosinophil growth factor. On the other hand *S. mansoni* infected-mice, treated with monoclonal antibody to IL-5 showed complete ablation of the eosinophil response to schistosomula migrating through skin (Sher, Coffman, Hieny & Cheever, 1990) indicating that IL-5 is the principal cytokine responsible for eosinophil production *in vivo*. The above results provide additional evidence for the SCF dependency of

parasitic infection-associated mast cell hyperplasia while demonstrating that SCF is not a significant factor in the associated eosinophilia.

The results described in chapter 7 showed that treatment of both normal and *N. brasiliensis* or *T. spiralis*-infected rats with polyclonal anti-SCF antibody significantly depressed mature mast cell numbers and jejunal and serum RMCP II concentrations, as well as parasite-associated mast cell hyperplasia (chapter 7). Similarly, in the experiment described here, mast cell densities and serum protease concentrations were almost completely ablated. However, in contrast with the intestinal nematode infections, where the mast cell numbers and protease levels had begun to increase again by as little as 6 days after the start of antibody treatment (chapter 7), the mast cell numbers and serum RMCP II levels in *S. mansoni*-infected rats remained depressed after 11 days of treatment with anti-SCF antibodies. This lack of any rebound response in the mast cells may simply be due to a lower level of antibody response to sheep IgG, although that was not measured in this study. It could also indicate that there is a different mechanism controlling mast cell hyperplasia in schistosomiasis than in the intestinal nematode infections, not involving T cell-derived cytokines such as IL-3. The role of IL-3 and other T cell-derived cytokines in the mast cell hyperplasia associated with schistosomiasis is the subject of continuing study.

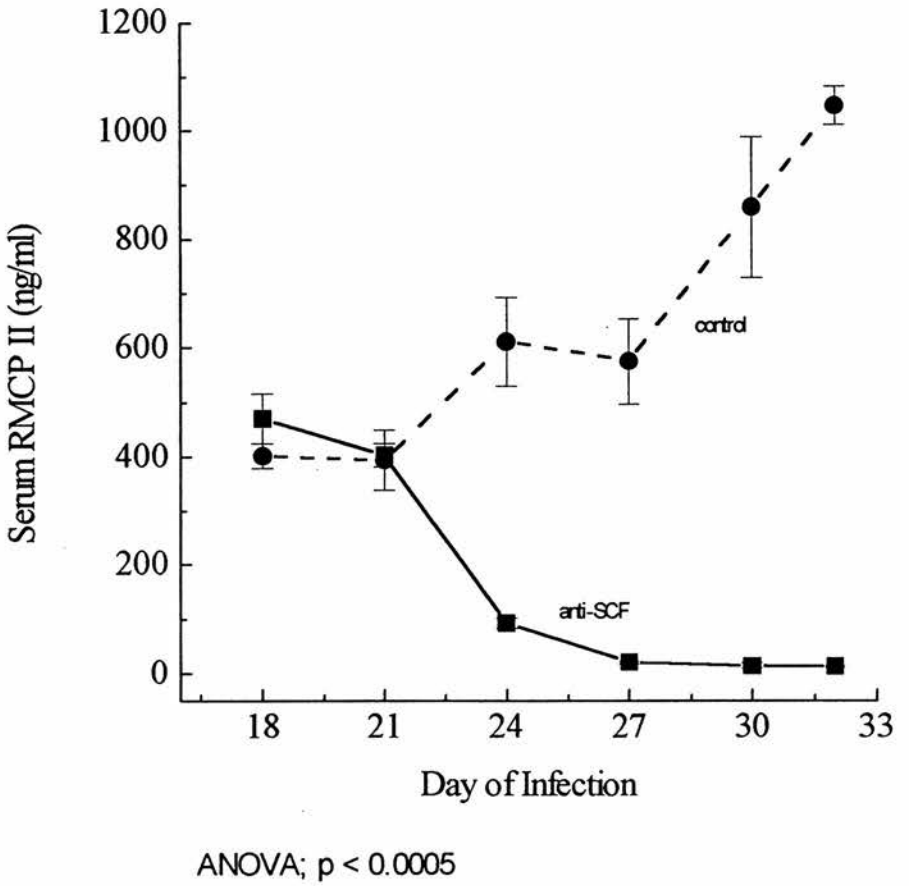


Figure 21. RMCP II concentrations in serum of *S. mansoni*-infected rats treated with anti-SCF or normal sheep IgG.

Table 18. Tissue mast cell protease concentrations and mast cell counts on day 32 of infection

.	RMCP I	RMCP II	mast cells	eosinophils
Jejunum (anti-SCF)	0**	20.2 ± 6.2***	1.24 ± 0.37***	60.47 ± 5.44
Jejunum (control)	0.89 ± 0.128	524.0 ± 35.86	49.76 ± 3.15	66.35 ± 3.30
Liver (anti-SCF)	0.69 ± 0.14***	10.5 ± 2.48**	0.27 ± 0.11***	33.74 ± 5.92
Liver (control)	5.58 ± 0.62	260.0 ± 73.31	7.61 ± 1.02	44.56 ± 4.98

Mast cell protease concentrations expressed in μg/g wet wt. of tissue.

cell counts expressed as cells/0.2 mm^2

Student's t-test, ** $p < 0.01$, *** $p < 0.0001$ comparing tissues from anti-SCF treated with control animals.

9. SOLUBLE STEM CELL FACTOR IS SECRETED INTO BLOOD OF NEMATODE-INFECTED RATS.

9.1 Introduction

Infection with gastrointestinal parasites induces a substantial hyperplasia of mast cells in the intestinal mucosa (Miller & Jarrett 1971). This hyperplasia is regulated through at least two distinct mechanisms; by T cell-derived cytokines like IL-3, 4, 9 and 10 (Madden *et al* 1991; Schmitt *et al* 1990; Thompson-Snipes *et al* 1991) and by SCF/*c-kit* (Chapters 5 - 8; Newlands *et al* 1995; Grencis *et al* 1993). The involvement of SCF and *c-kit* in mast cell hyperplasia is clearly demonstrated by the ablation of the intestinal mast cell response in parasitised rodents treated with antibodies directed against either *c-kit* or SCF (Grencis *et al* 1993; Donaldson, Schmitt, Huntley, Newlands & Grencis, 1996; Chapters 6 - 8; Newlands *et al* 1995). In man soluble SCF is present in blood at concentrations of around 3 $ng.ml^{-1}$ as measured by enzyme linked immunosorbent assay (ELISA) of sera from normal individuals (Langley, Bennett, Wypych, Yancik, Liu, Westcott, Chang, Smith & Zsebo, 1993). In patients with aplastic anaemia the range of serum SCF concentrations is similar to that found in normal individuals (0.3 - 6.1 $ng.ml^{-1}$) but those with the most severe clinical symptoms tend to have lower concentrations of SCF, the majority being below the mean normal value of 3.3 $ng.ml^{-1}$ (Wodnar-Filipowicz, Yancik, Moser, Dalle-Carbonare, Gratwohl, Tichelli, Speck & Nissen, 1993). Thus the measurement of SCF in the blood of rats undergoing active mast cell hyperplasia, in which SCF is known to play an important role, would be invaluable in the study of the kinetics of the SCF response to parasitic infection. In the work

described in this chapter a highly sensitive ELISA test was developed to measure soluble SCF in the serum of normal or parasitised rats as well as rats which were treated with either rrSCF[164] or with polyclonal antibody to SCF. The results presented here show that SCF can be detected in normal rat serum and that levels are substantially up-regulated by nematode infection.

9.2 Experimental Design.

The aims of the work described in this chapter were first to develop an assay to measure physiological concentrations of SCF in the sera of rats and secondly to study the kinetics of the SCF response in parasitised rats.

9.2.1 Optimisation of SCF ELISA.

The format of the ELISA was that of an antigen capture-type test. Detection was with a biotinylated secondary antibody followed by streptavidin-HRPO (Boehringer-Mannheim, U.K.).

The capture antibody, affinity purified polyclonal sheep anti-SCF, was coated onto the test plates (Dynatech M129B) at the rate of 0.1, 0.5, 1.0, 5.0 or 10.0 $\mu g.ml^{-1}$ in 0.1 M carbonate buffer pH 9.6.

The optimum dilution of the secondary antibody, biotinylated, sheep anti-SCF for the standard concentration range, was determined by titration of the 1 $mg.ml^{-1}$ stock at 1/125, 1/250, 1/500 and 1/1000. The final steps in the titration of both capture and secondary antibodies was the addition of streptavidin-HRPO, standardised at a dilution of 1/500 in accordance with the manufacturer's recommendation, and utilising orthophenylenediamine (Sigma, Poole, UK) at a

concentration of 0.4 $mg.ml^{-1}$ and H_2O_2 (60 µl/10 ml) as substrate. Plates were read at 492 nm on a Titertek MC ELISA plate reader (Titertek-Flow, Paisley, UK).

Samples and standards were prepared by dilution with 5% $^{w}/_{v}$ bovine serum albumin (Sigma) in phosphate buffered saline (BSA/PBS). Serum samples were diluted 1:20 with BSA/PBS before assay. Standard curves were prepared which covered the range 0.5 pg to 20 $ng.ml^{-1}$. In order to determine whether the assay would detect physiological concentrations of SCF in serum, normal rat serum was spiked with $rrSCF^{164}$ at a concentration of 1 $\mu g.ml^{-1}$. A number of dilutions of this spiked serum, in the range 5 to 750 $pg.ml^{-1}$ were prepared in BSA/PBS for assay.

The different coating antibody densities gave a concentration dependent increase in absorbance at 492 nm up to 1.0 $\mu g.ml^{-1}$, with a standard curve which covered the range 5 to 750 $pg.ml^{-1}$ (Figure 22a), and did not increase significantly at higher concentrations. Further optimisation of the test was therefore based on plates coated at 1.0 $\mu g.ml^{-1}$.

Dilutions of biotinylated sheep anti-SCF at 1/125, 1/250 and 1/500 gave very similar results, with somewhat lower absorbances for the 1/1000 dilution (Figure 22b). A 1/500 dilution of the biotinylated, sheep anti-SCF, secondary antibody was therefore used in later tests.

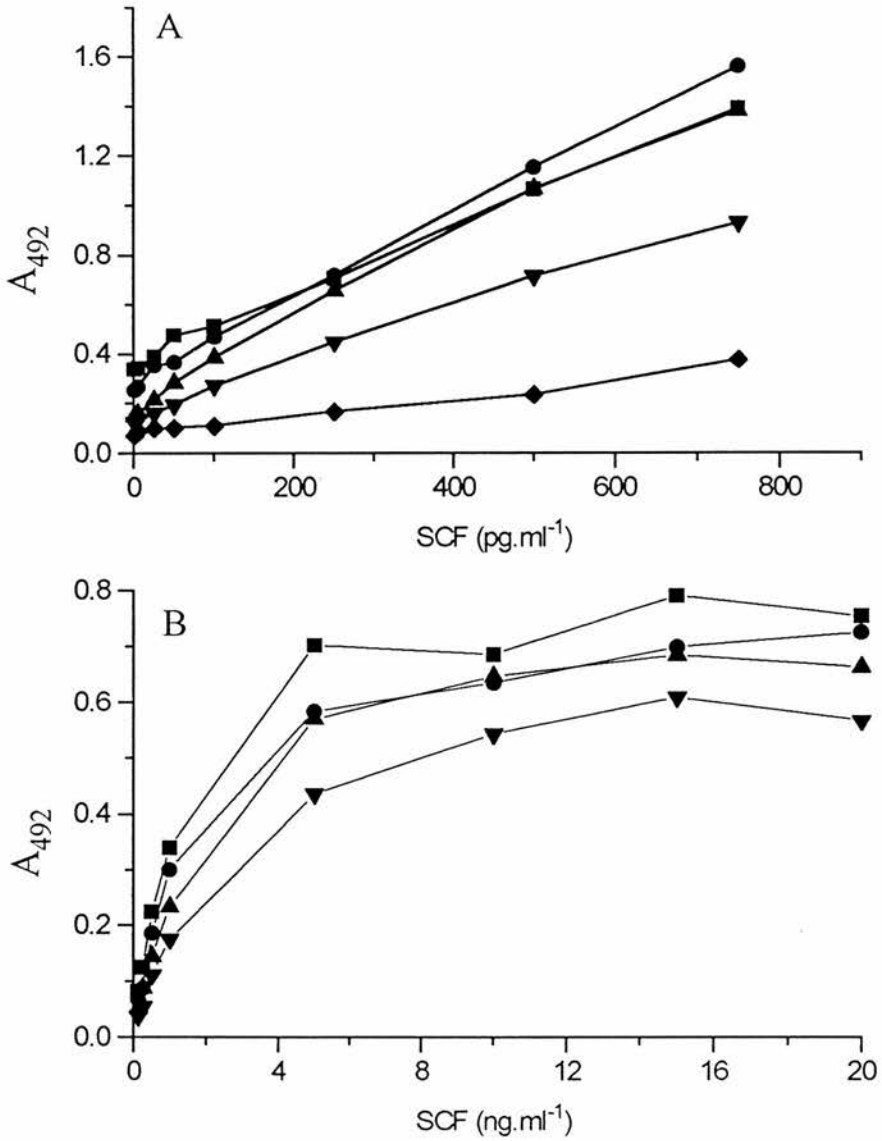


Figure 22. Optimisation of the SCF ELISA.
The graphs show the effects on A_{492} of varying (A) the capture antibody concentration (0.1 μg.ml^{-1} ,◆; 0.5 μg.ml^{-1} ,▼; 1.0 μg.ml^{-1} ,▲; 5.0 μg.ml^{-1} ,● or 10.0 μg.ml^{-1} ,■) or (B) the concentration of the streptavidin-HRPO conjugate (1:125, ■; 1:250, ●; 1:500,▲ or 1:1000, ▼).

9.2.2 Experiment 1.

To determine the effects of administration of exogenous SCF on circulating soluble SCF concentrations, recombinant rat stem cell factor[164] , modified by the covalent attachment of polyethylene glycol (Tsai *et al* 1991a), was administered intravenously to anaesthetised rats for 14 consecutive days in 1 ml physiological saline at 25μg/kg of body weight/day, control rats were sham treated with physiological saline alone. The rats were killed 24 h after the final injection. The protocols were the same as those described previously (Tsai *et al* 1991a; Newlands *et al* 1995; Chapter 5) and all blood samples were derived from these same sets of experiments. The mast cell responses have therefore been described previously (Tsai *et al* 1991a; Newlands *et al* 1995; chapter 5).

The blood volume of rats weighing approximately 250g has been estimated at 50 ml/kg of bodyweight and the plasma volume at 30 ml/kg of bodyweight (Schalm *et al* 1975); thus the SCF dose/ml/day can be calculated from the following :-

Equation 10

$$\frac{\text{Dose (μg/kg bodyweight) x bodyweight (kg)}}{\text{Serum volume (ml/kg bodyweight) x bodyweight (kg)}} = \frac{25 \times 0.25}{30 \times 0.25} = 0.833\ \mu g.ml^{-1} \text{ of plasma}$$

9.2.3 Experiment 2.

To determine the effects of anti-SCF antibody treatment on circulating SCF concentrations rats were inoculated with 1 mg sheep anti-rrSCF[164] or normal sheep IgG in 1 ml phosphate buffered saline (PBS) by intraperitoneal injection. Normal rats

were treated daily for each of 4 consecutive days, as described (Newlands *et al* 1995; Chapter 6), to determine the effects on baseline serum SCF concentrations. Rats infected with *N. brasiliensis* were treated with anti-rrSCF^{164} on days 3, 5, 7, 10 and 12 of infection. For rats infected with *T. spiralis,* treatment commenced on day 0 and continued on days 3, 5, 7 and 10. Normal rats were killed 24 hours, and parasitised rats 48 hours, after the final inoculation (Newlands *et al* 1995; Chapters 6 and 7). Blood was collected, allowed to clot at room temperature and the serum subsequently harvested and stored at -20°C before assay.

9.2.4 Data presentation and analysis

Unless otherwise specified, all data are presented as the mean ± s.e.m. The 2-tailed Student's t-test was used to analyse data that were normally distributed, whereas the Mann-Whitney U test was used as the non-parametric test. The time course of responses in different groups of rats were examined for statistical significance by using analysis of variance (ANOVA). $p < 0.05$ was taken to indicate a significant difference.

9.3 Results

Standard curves, diluted with BSA/PBS, showed that the assay had a functional range of 1 pg.ml^{-1} to 10 ng.ml^{-1} (Figure 23a and Figure 23b). Assay of normal rat serum spiked with rrSCF^{164} detected concentrations of SCF which, when compared with the values obtained for the standard curve, diluted with BSA/PBS, gave a highly significant correlation ($R^2 = 0.99$; Figure 23c).

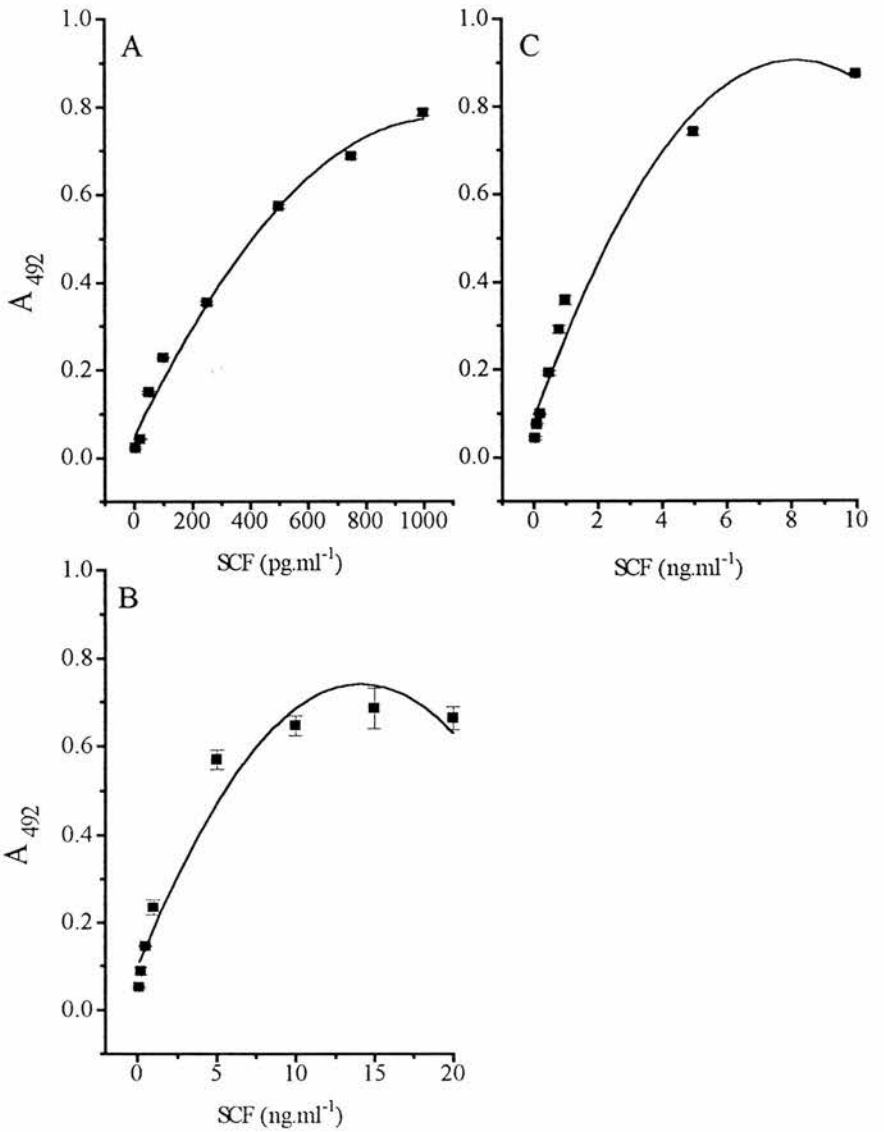


Figure 23. SCF ELISA standard curves.

Standard curves were prepared by diluting rrSCF^{164} with PBS/BSA to cover the ranges (A) 0.5 to 1000 pg.ml^{-1} and (B) 125 pg.ml^{-1} to 20 ng.ml^{-1}. (C) Standard curve prepared from normal rat serum spiked with rrSCF^{164} to a concentration of 1.0 μg.ml^{-1} and subsequently diluted with PBS/BSA to cover the range 50 pg.ml^{-1} to 10 ng.ml^{-1} and compared with standards diluted with PBS/BSA alone. The A_{492} values are shown as closed squares with error bars and the fitted curve for the standards prepared in PBS/BSA is shown as a line for comparison.

9.3.1 SCF concentrations in the blood of rats given rrSCF^{164} by daily intravenous injection.

The estimated daily dose of SCF/ml of serum is 833 ng.ml^{-1}, calculated from Equation 10. Serum samples, collected 24 hours after the final injection of rrSCF^{164}, from normal rats which had been treated by daily intravenous injection (n = 8) had 9.0 ± 0.63 ng.ml^{-1} of SCF and therefore the SCF content of serum had decreased to around 1% of the daily dose. Control rats, which had been given vehicle (PBS) only, had 140 ± 140 pg.ml^{-1} (n = 8).

9.3.2 Soluble SCF concentrations in the blood of normal and parasitised rats.

The concentrations of SCF detected in the serum of normal rats, before infection with *N. brasiliensis* (n = 10) was in the range 254 - 875 pg.ml^{-1} with a mean of 578 ± 65 pg.ml^{-1} (Figure 24a). After infection there was a slight, but significant, decrease in soluble SCF at day 6 compared with pre-infection values ($p < 0.05$), followed by a steady increase to 1800 pg.ml^{-1} at day 14, a three-fold increase compared with pre-infection controls (Figure 24a). Over the course of the infection the increase in soluble SCF was highly significant by ANOVA ($p<0.0001$).
Rats in the group infected with *T. spiralis* (n = 10) had 337 ± 76 pg.ml^{-1} of serum before infection and there was a significant increase ($p < 0.005$) on day 6 compared with pre-infection values (Figure 24a). The SCF concentration continued to rise sharply to 3831 pg.ml^{-1} by day 10 of infection, an increase of 11 fold compared with pre-infection controls, and then declined to 1561 pg.ml^{-1} by day 12 (Figure 24a).

These changes were significant by ANOVA ($p<0.05$) over the course of infection with *T. spiralis.*

9.3.3 The effects of anti-SCF treatment on concentrations of SCF in serum from normal and parasitised rats.

The concentration of soluble SCF in serum from normal Wistar rats was 471 ± 57 $pg.ml^{-1}$ ($n = 18$) at the start of the experiment. After 4 days treatment with polyclonal sheep anti-SCF ($n = 5$) the concentrations of SCF in serum were below the level of detection for the assay ($p < 0.001$) compared with pre-infection controls. Following infection with *N. brasiliensis*, anti-SCF-treated rats showed a three-fold decrease in serum SCF compared with pre-infection values (Figure 24b; $p = 0.001$). This was followed by a sharp increase, to 5829 $pg.ml^{-1}$, >10 fold more than pre-infection values at day 10, before falling back to 2400 $pg.ml^{-1}$ by day 14 (Figure 24b). Over the course of the infection the changes in SCF concentration were highly significant by ANOVA ($p = 0.002$). Anti-SCF-treated rats which were infected with *T. spiralis* did not show the initial decrease in serum SCF at day 6 which was seen in *N. brasiliensis*-infected animals (Figure 24b). By day 10 SCF concentrations were increased by 4 fold compared with pre-infection values (Figure 24b) and, as in *N. brasiliensis*-infected animals, the concentration fell again by day 12 (Figure 24b). These changes in SCF concentrations were also highly significant by ANOVA ($p < 0.001$) over the course of the infection.

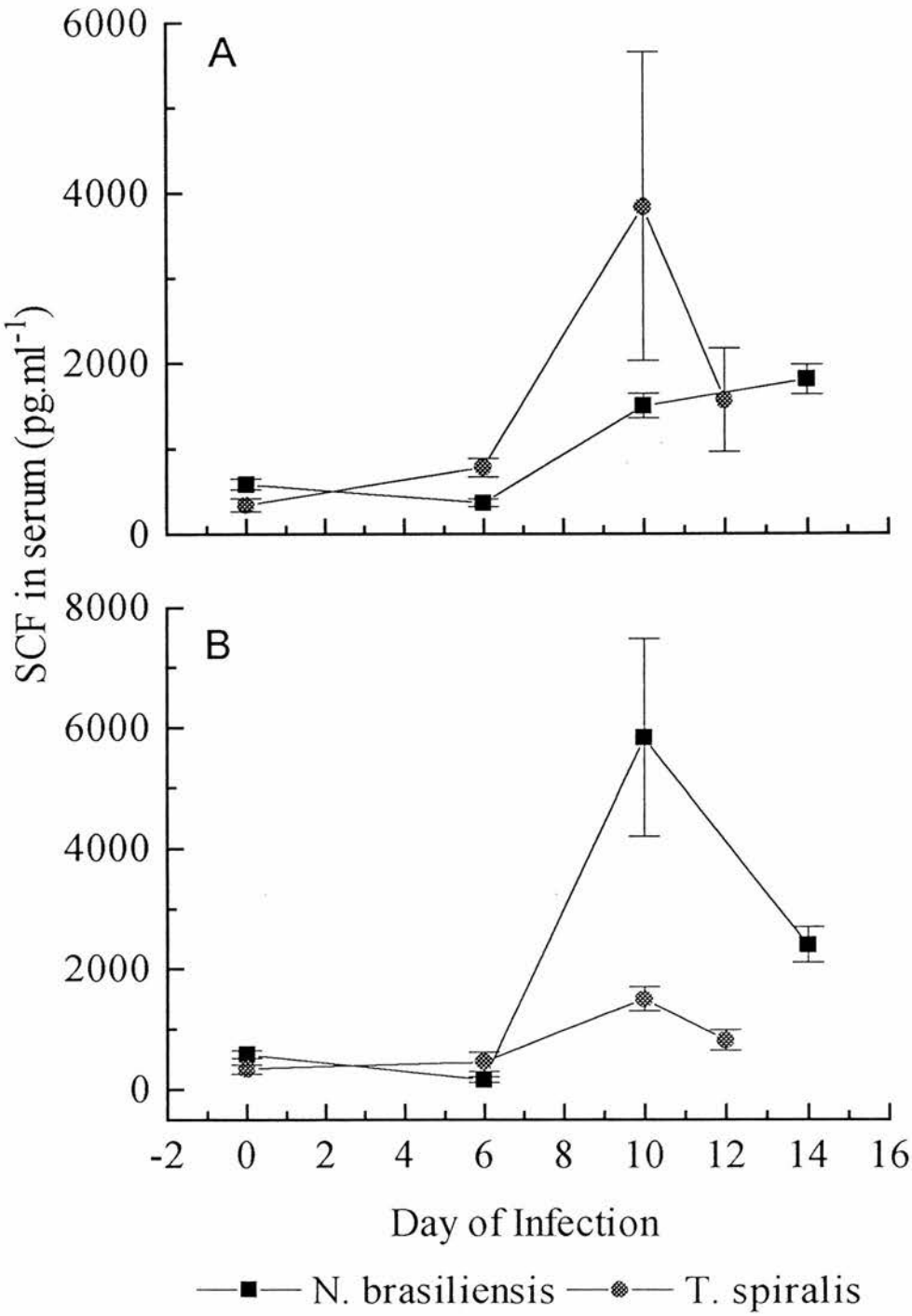


Figure 24. Concentration of SCF in the sera of *N. brasiliensis* or *T. spiralis*-infected rats treated with (A) control Ig or (B) anti-SCF antibodies.

9.4 Discussion

SCF is expressed in both membrane-bound and soluble forms (Anderson *et al* 1990) and in man the concentration of the soluble form has been measured at 3.3 ng. ml^{-1} by ELISA in the sera of normal individuals (Langley *et al* 1993). In the results described here the concentrations of soluble SCF detected in the sera of normal Wistar rats was 578 $pg.ml^{-1}$, which is almost six-fold less than that detected in man.

The ELISA test described here is highly sensitive, detecting SCF in pg concentrations in serum. The assay gives highly reproducible results over concentration ranges from 5 $pg.ml^{-1}$ to 20 $ng.ml^{-1}$ with correlation coefficients of 0.96 or greater for the relationship between SCF concentration and absorbance (Figure 23).

Measurement of SCF in the blood of rats which were given $rrSCF^{164}$ by intravenous injection revealed that only 1% of the daily dose could be detected 24 hours after the final injection. It is demonstrated here that $rrSCF^{164}$ added to serum can be fully detected by the ELISA (Figure 23c) and therefore the decrease in circulating levels of SCF is probably due to its removal from the circulation. The receptor for SCF, encoded for by the *c-kit* proto-oncogene is a trans-membrane tyrosine kinase (Matsui, Zsebo & Hogan, 1990) which will bind free SCF. The mast cell proliferation which takes place both locally, at the site of injection, and systemically following treatment with $rrSCF^{164}$ (Tsai *et al* 1991a) suggests that the injected cytokine is binding to this receptor *in vivo*. In man there is also a soluble form of *c-kit* receptor, present in 100 fold excess over reported concentrations of SCF; 324

$ng.ml^{-1}$ kit receptor (Wypych, Bennett, Schwartz, Clogston, Broudy, Bartley, Parker & Langley, 1995) to 3.3 $ng.ml^{-1}$ for SCF (Langley *et al* 1993) which may be responsible for the complexing and subsequent removal of excess free SCF.

In chapter 7 it was demonstrated that rats infected with *N. brasiliensis* or *T. spiralis*, showed the expected pattern of intestinal mast cell hyperplasia associated with gastrointestinal parasitism (Newlands *et al* 1995). In addition, similarly infected rats, which were treated with polyclonal sheep anti-SCF antibodies, show a delay in mast cell hyperplasia with no mast cells detected at day 10 of infection with either parasite, followed by a sharp increase in mast cell numbers (chapter 7; Newlands *et al* 1995). In this chapter the SCF concentrations in the sera of these same rats was examined and it is clear that there is a significant increase in the concentrations of soluble SCF in blood after day 10 of infection with both *N. brasiliensis* or *T. spiralis* infections which coincides with the observed intestinal mast cell hyperplasia in these animals (chapter 7; Newlands *et al* 1995).

In rats treated with anti-SCF antibodies the intestinal mast cell hyperplasia seen in both *N. brasiliensis* and *T. spiralis* infections was almost completely suppressed at day 10 of infection (Newlands *et al* 1995). In contrast, whilst there were almost no mast cells detected in the intestinal mucosa of rats at day 10 of infection with either parasite (Newlands *et al* 1995), there are significantly increased concentrations of SCF (Figure 24b). Although the comparisons must be treated with some caution because they are from separate experiments, the increase in SCF at day 10 in anti-SCF-treated rats appears to be greater in *N. brasiliensis*-infected than *T. spiralis*-infected rats (Figure 24b). This may be because the mast cell response to *T. spiralis*

is less dependent on SCF than that directed against *N. brasiliensis*. The response to *T. spiralis* does require some SCF in the initial stages since the hyperplasia is blocked with antibody (Newlands *et al* 1995; Donaldson *et al* 1996) but the later stages may be more dependent on IL-3 and IL-4 since treatment with antibodies to these cytokines also blocks mast cell hyperplasia in mice (Madden *et al* 1991). It is, perhaps, surprising that there is an increase in detectable SCF after treatment with anti-SCF antibodies and several possible explanations suggest themselves. It may be that because mucosal mast cell hyperplasia has been blocked there are simply fewer receptor sites around to which the SCF can bind. An alternative explanation may be that SCF secretion is regulated through a feed-back loop where a decrease in the concentrations of circulating SCF, for example by being complexed to antibody, stimulates up-regulation of expression.

In summary the assay described here allows the kinetics of expression of the soluble factor to be followed in detail and since the soluble and membrane-bound forms of SCF are encoded for by the same gene, this may reflect the total expression of this potent haemopoietic growth factor.

10. GENERAL DISCUSSION.

Mast cell hyperplasia is a prominent feature of the host's immune response to gastrointestinal helminth parasites and, is associated with increased concentrations of total and of parasite-specific IgE (Jarrett & Miller 1981). The activation of mast cells through IgE-mediated immediate hypersensitivity reactions may be important in protection against certain nematode parasites. Mast cells are a source of many potent mediators of immune and inflammatory processes. In this thesis the glycoforms and activity of one of these mediators, the secretory granule-associated chymase, MMCP-1, in the mouse are examined. This study was followed with an examination of the mechanisms regulating both mast cell protease expression and the survival, growth and proliferation of mast cells in normal and parasitised animals.

The studies of murine chymases revealed that not only is there a diversity of proteases synthesised by murine mast cells (Newlands *et al* 1987; Reynolds *et al* 1990; McNeil *et al* 1992) but also that the first identified of these, MMCP-1 (Newlands *et al* 1987) is synthesised in at least 5 different glycoforms (chapter 3). These glycoforms were identified as variants of MMCP-1 by a variety of techniques, including N-terminal sequencing, and were shown to have different enzymic properties against low molecular weight substrates (chapter 4). The major questions raised by these findings are:

1) Do the different glycoforms represent genuine variants of the protease or do they simply represent a number of intermediates in the glycosylation process? This is probably best addressed by analysis of the structure of the carbohydrate moieties of each of the variants. This would provide valuable information on whether there is an

identifiable series of intermediates which would fit into a known series of steps in the processing of carbohydrate side chains.

2) Are the different glycoform synthesised by different sub-sets of the MMC population depending on their anatomical distribution or indeed by mast cells at different stages of maturation? To identify whether there is a differential distribution of the glycoforms it would be necessary to develop a panel of monoclonal antibodies which were specific for the carbohydrate moieties, since the variants all share the same core polypeptide. This may prove difficult as carbohydrate epitopes are notoriously heterogeneous and often shared by many proteins. Alternatively it may be possible to characterise the carbohydrates through the use of a panel of lectins.

3) If the glycoforms do represent variants of the mature enzyme, what is their function *in vivo* and what are their native substrates? RMCP II, the rat MMC protease increases vascular and epithelial permeability (Scudamore *et al* 1995) either by proteolysis of epithelial tight junction proteins between cells or through proteolysis of thrombin-type protease-activated receptors. It would be very interesting to compare the enzymic properties of the MMCP-1 variants using tight junction components such as occludin as substrates.

The regulation of mast cell protease expression has been investigated *in vitro* in both BMMC, generated from bone marrow and in CTMC derived from the peritoneal cavity. When bone marrow from rats or mice is cultured in the presence of IL-3 or T cell conditioned medium mast cells are produced which, in the rat, closely resemble MMC in terms of morphology, staining properties etc. and more specifically that they express abundant RMCP II (Haig *et al* 1988). Cultured murine BMMC are, however

heterogeneous in terms of their protease content with protease phenotypes resembling both MMC and CTMC (Newlands *et al* 1991). Indeed when murine BMMC are grown in IL-3 alone they do not express MMCP-1 unless they are concomitantly or sequentially exposed to IL-10 (Ghildyal *et al* 1992). By contrast, rat CTMC are IL-3-independent and do not proliferate significantly when cultured with this cytokine alone (Haig *et al* 1994). However, when they are cultured in the presence of SCF, CTMC proliferate rapidly (Tsai *et al* 1991b; Haig *et al* 1994). On addition of IL-3 to the culture medium the two factors work in synergy to increase the rate of mast cell growth (Haig *et al* 1994). An additional effect of this treatment is that it induces CTMC to begin expressing RMCP II (Haig *et al* 1994). All these data are derived from *in vitro* culture experiments and little is known about the *in vivo* regulatory mechanisms. The effects of exogenous cytokines on mast cell populations *in vivo* were therefore examined.

Intravenous or intraperitoneal treatment of rats with rrSCF^{164} , which has high biological activity (Galli *et al* 1994), confirmed the *in vitro* finding that SCF provides a potent proliferative signal for CTMC but that it suppressed protease expression (chapter 5). It is interesting to note that while intravenous SCF did not promote RMCP II expression in CTMC the direct delivery of SCF into the peritoneal cavity did (chapter 5), suggesting that the local concentration of SCF may be important to generate the synergistic effect with the background concentrations of IL-3. This could be readily tested in an *in vivo* experiment where groups of rats were treated with a range of different concentrations of SCF.

LNCM, a rich source of IL-3, which is a potent, *in vitro* growth factor for BMMC, did not stimulate an increase in the numbers of CTMC but did induce them to start expressing RMCP II (chapter 5), an effect which was increased by the addition of SCF. Thus it is clear that both SCF and IL-3 are closely involved in the *in vivo* regulation of mast cell chymases in the rat.

In order to further investigate the role of SCF in the regulation of mast cell populations in the rat a sheep polyclonal antibody to rrSCF[164] was developed. This reagent was initially used *in vivo* to block the effects of SCF on mast cell populations in normal rats (chapter 6). The results of this treatment were that there was a 50% depletion of CTMC from the peritoneal cavity indicating that either a sub-set of peritoneal cavity CTMC are dependent on SCF for their *in vivo* survival or that the dosage rate for the antibody was inadequate (chapter 6). The former explanation seems more likely since, in the same rats, this treatment results in complete ablation of MMC from the small intestinal mucosa (chapter 6). This is probably as a result of the mast cells undergoing apoptosis in the absence of SCF (Iemura *et al* 1994). There was also a moderate depletion of leukocytes from blood. This appeared to be a generalised effect since the relative proportions of white blood cells, neutrophils, lymphocytes, monocytes etc. remained constant in both anti-SCF treated and control rats. (chapter 6). To more fully investigate the effects of this type of treatment on the haemopoietic system, it would be necessary to undertake in-depth studies of bone marrow stem cells, which also bear the *c-kit* receptor for SCF.

The almost total ablation of MMC in normal intestine was somewhat unexpected and showed that SCF has a more important role in maintaining the

viability of MMC than had been formerly thought. To extend the findings that SCF is essential for MMC survival, its role in mast cell proliferation was examined in parasitised rats which undergo substantial mast cell hyperplasia as part of the immune response to the parasites. Rats infected with *N. brasiliensis* or *T. spiralis* were treated with anti-SCF antibodies throughout the course of infection and in both experiments the expected MMC hyperplasia was significantly delayed but not completely abrogated since both mast cell numbers and RMCP II concentrations in blood had reached near normal levels by the end of the experiments (chapter 7). Interestingly, even when antibody treatment was not started until day 10 of infection, by which time the MMC hyperplasia was well established, there was still a significant depletion in mast cell numbers when compared with controls (chapter 7). This demonstrates that even when there is a predominantly T cell-driven hyperplasia (athymic 'nude' mice do not develop MMC hyperplasia [Reed, 1985]) these mast cells are, at least in part, SCF dependent.

The development of a mast cell hyperplasia in parasitised rats, treated with polyclonal sheep anti-SCF antibodies (chapter 7) may have several causes. It is clear that the rats used in these experiments rapidly developed an antibody response to the sheep immunoglobulin (chapter 7) which would, presumably, reduce the efficacy of the treatment and thus allow the SCF to fulfil its function. On the other hand, in rats treated with anti-SCF antibodies there is at least a two-fold increase in the concentrations of circulating, soluble SCF when compared with controls treated with normal sheep IgG (chapter 9). It seems likely, therefore, that there is a regulatory feed-back mechanism which up-regulates SCF production in response to low

concentrations of soluble SCF in blood. SCF is expressed in both soluble and membrane-bound forms (Anderson *et al* 1990) and it is therefore difficult to estimate the contribution of the membrane-bound product to mast cell hyperplasia. Assaying the soluble product in blood by ELISA may not reflect the level of up-regulation of the membrane-bound form since production of soluble SCF is dependent on release of the extracellular domain by proteolytic processing (Anderson *et al* 1990). This could be addressed by an immuno-histochemical study aimed at identifying which cells express surface SCF and perhaps obtaining a semi-quantitative measurement of membrane-bound SCF by image analysis. There may also be a strain-dependent component to the recovery of the mast cell response since F344 rats, infected with *S. mansoni* showed a complete ablation of intestinal mast cells following anti-SCF treatment and did not recover by the end of the experiment 12 days later (chapter 8).

It is also interesting to note that, in the rats which had their MMC hyperplasia suppressed by anti-SCF treatment, the female worms had a lower level of fecundity compared with those in control rats (chapter 7). This is the opposite effect to that which might have been expected if the mast cell response was a significant part of the protective immune response against the parasite. This is similar to the effects found when mice, infected with *S. mansoni,* were treated with anti-IgE antibodies (Amiri *et al* 1994). Mice treated in this way had fewer worms and there were fewer eggs produced per worm (Amiri *et al* 1994). This suggests that the parasites in these infection models have evolved to cope with the hostile environment provided by their host and that, in fact, the activation of mast cells through parasite-specific IgE may facilitate their survival.

In conclusion, therefore the mast cell response may, under certain circumstances, favour parasite survival and under others promote worm expulsion. Based on the recent studies by Scudamore *et al* (1995) the soluble chymases RMCP II and MMCP-1 may promote epithelial permeability and this in turn will facilitate translocation of plasma proteins into the gut lumen. Such a mechanism may initially prove favourable to lumen-dwelling parasites like *N. brasiliensis* but as parasite-specific antibodies become more abundant the mast cell-mediated leak lesion may promote a less favourable environment for worm survival.

Since it is increasingly recognised that mast cell proteases can regulate cell proliferation and production of cytokines like IL-8, probably via thrombin-type protease-activated receptors (Cairns & Walls, 1996), the abundance, specificity and function of mast cell chymases and tryptases and their different glycoforms will be of increasing interest.

In this thesis and in ongoing work with my collaborators it is clear that chymase expression is modulated by cytokines and that a better understanding of the mechanisms regulating the tissue-specific expression of the soluble MMC chymases MMCP-1 and RMCP II and their functions at mucosal surfaces may provide important insights into the function of MMC.

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12. APPENDIX.

12.1 Anti-stem cell factor treatment: Effects on peritoneal mast cell populations in parasitised rats.

These are the results, from the experiments described in chapter 7, of the effects of anti-SCF treatment on peritoneal mast cell populations in *N. brasiliensis* or *T. spiralis*-infected rats.

12.1.1.1 Effects in *N. brasiliensis*-infected rats.

Infection with *N. brasiliensis* increased the total number of cells recovered by peritoneal lavage from control rats treated with normal sheep immunoglobulins, at days 6 and 10 of infection, compared with baseline values (Table 10, chapter 6; Table 19). There was, however, no significant difference between infected rats treated with control IgG or anti-SCF antibodies (Table 19). The number of mast cells recovered by lavage from the anti-SCF-treated rats, over the course of infection was significantly decreased compared with controls ($p = 0.003$ by ANOVA; Table 19) but the RMCP I content of these cells was unaltered except for a slight decrease in the controls at day 6 compared with baseline values reported in chapter 6 ($p < 0.05$; Table 19, Table 10).

In this experiment, infection with *N. brasiliensis* was accompanied by a slight increase in mast cell RMCP II content of > 4-fold over baseline values. This was in sharp contrast with the previous experiment where *N. brasiliensis* infection caused a 340-fold increase in RMCP II (Table 19, Table 8). However, even this relatively slight increase in RMCP II content was completely abolished by treatment with anti-SCF ($p = 0.026$, Table 19). The relatively low RMCP II levels in the controls was reflected in the percentage of mast cells which stained for the presence of RMCP II by

immunofluorescence. The fluorescence was maximal at day 10 of infection when 5.8% of the detected mast cells stained RMCP II^+ (Table 19). This immuno-fluorescence was almost completely abolished by anti-SCF treatment (Table 19).

Table 19; Effects of anti-SCF treatment on peritoneal mast cells of *N. brasiliensis*-infected rats.

	n	Total cell / lavage ($x10^7$)	total mast cells/lavage ($x10^6$)	RMCP I (pg/mast cell)	RMCP II (fg/mast cell)	Total % RMCP II+ cells
D 6 control	5	4.5 ± 0.57	1.1 ± 0.30	74.0 ± 12.81	17.9 ± 15.03	1.4 ± 0.88
D 10 control	5	3.2 ± 0.43	0.7 ± 0.15	142.7 ± 24.66	25.7 ± 4.69	5.8 ± 0.97
D 14 control	5	3.1 ± 0.59	0.62 ± 0.11	106.2 ± 26.52	26.2 ± 2.76	0.3 ± 0.12
D 6 anti-SCF	5	3.0 ± 0.47	0.5 ± 0.11	149.3 ± 26.03 *	7.9 ± 2.88	$0.1 \pm 0.10^{+}$
D 10 anti-SCF	5	2.8 ± 0.29	0.3 ± 0.13	100.7 ± 26.66	13.9 ± 5.21	$0.4 \pm 0.33^{++}$
D 14 anti-SCF	5	3.1 ± 0.33	0.25 ± 0.12	138.3 ± 37.98	9.1 ± 2.85 **	0.0 ± 0.00
ANOVA		NS	p = 0.003	NS	p = 0.026	p = 0.001

Student's two sample t-test; * $p < 0.05$, ** $p < 0.01$.

Mann-Whitney non-parametric test; $^{+}$ $P < 0.05$, $^{++}$ $P < 0.01$

ANOVA; control time course vs. anti-SCF-treated time course.

Table 20; Effects of anti-SCF treatment on peritoneal mast cells of *T. spiralis*-infected rats.

	Day of Infection	n	Total cells/Lavage ($x10^7$)	Mast Cells/Lavage ($x10^6$)	RMCP I (pg/Mast Cell)	RMCP II fg/Mast Cell)
control	6	4	4.5 ± 0.18	0.8 ± 0.14	138 ± 9.0	10 ± 1.6
	10	5	4.5 ± 0.18	1.0 ± 0.15	153 ± 16.0	43 ± 8.6
	12	5	4.5 ± 0.22	0.8 ± 0.13	103 ± 18.0	23 ± 4.7
anti-SCF	6	5	3.4 ± 0.11 **	0.43 ± 0.03	200 ± 34.0	3 ± 0.2 *
	10	5	3.1 ± 0.41 *	0.1 ± 0.04 **	579 ± 174.0	18 ± 4.2
	12	5	3.9 ± 0.26	0.2 ± 0.04 *	197 ± 30.0 *	21 ± 4.1
ANOVA			$p < 0.0005$	$p < 0.0005$	$p = 0.008$	$p = 0.018$

Student's two sample t-test, * $p < 0.05$, ** $p < 0.01$.

12.1.1.2 Effects in *T. spiralis*-infected rats.

The total number of cells recovered by lavage from the control rats, which were infected with *T. spiralis* and treated with normal sheep immunoglobulins, was increased compared with baseline levels (Table 20, Table 10). This increased cellularity was significantly decreased in the anti-SCF-treated group compared with controls ($p < 0.0005$ by ANOVA; Table 20). In this experiment infection with *T. spiralis* had no effect on serosal mast cell numbers in the control rats when compared with baseline values (Table 20, Table 10) whilst the rats treated with anti-SCF showed a marked depletion of mast cells, over the course of infection, compared with controls ($p < 0.0005$ by ANOVA, Table 20). The mean mast cell RMCP I content was not significantly altered by infection and treatment with control IgG whereas treatment with anti-SCF increased the levels of RMCP I substantially ($p = 0.008$, Table 20). Infection with *T. spiralis*, like *N. brasiliensis,* stimulated synthesis of RMCP II in the peritoneal mast cell population in both the control and anti-SCF-treated groups although there was less protease detected in the lavage obtained from the anti-SCF-treated group ($p = 0.018$, Table 20).

12.2 Discussion

Rats, infected with *N. brasiliensis* or *T. spiralis* and treated with anti-SCF both showed marked depletion of peritoneal mast cells and, in the *T. spiralis*-infected rats, the mean RMCP I content increased substantially. The increased content of RMCP I may be caused by the removal of down-regulating SCF by antibody or by some aspect of the potent T cell response generated against *T. spiralis* infection. The up-regulation of RMCP II expression, so marked in the earlier experiments (Table 7, Table 8; Chapter 5), was less pronounced, but still significant, in both *N. brasiliensis* and *T.*

spiralis infections where the rats were treated with control immunoglobulins (Table 19, Table 20). RMCP II production by serosal mast cells was significantly reduced, in both infection models, by treatment with anti-SCF (Table 19, Table 20). This clearly indicates that SCF is a necessary component in the up-regulation of RMCP II expression by serosal mast cells.

13. PAPERS ARISING FROM THIS THESIS.

Newlands, G. F. J., Knox, D. P., Pirie-shepherd, S. R. & Miller, H. R. (1993). biochemical and immunological characterization of multiple glycoforms of mouse mast cell protease 1: comparison with an isolated murine serosal mast cell protease (MMCP-4). *Biochem. J.* **294**: 127-35.

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Newlands, G.F.J., Coulson P.S. and Wilson R.A. (1995). Stem cell factor dependent hyperplasia of mucosal-type mast cells but not eosinophils in *Schistosoma mansoni*-infected rats. *Parasite Immunology* **17**: 595-598

Biochemical and immunological characterization of multiple glycoforms of mouse mast cell protease 1: comparison with an isolated murine serosal mast cell protease (MMCP-4)

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Five highly soluble, chymotrypsin-like, neutral serine proteases, with molecular masses in the range 30–33 kDa, were isolated from *Trichinella spiralis*-infected mouse small intestine. These enzymes were closely related antigenically on Western blotting and by Ouchterlony double diffusion using a polyclonal, cross-absorbed, sheep antibody raised against mouse mast cell protease-1 (MMCP-1), and, on the basis of N-terminal amino acid sequence analysis, were identified as variant forms of MMCP-1. Substrate and inhibitor analysis confirmed that the five variants (MMCP-1 A–E) had similar characteristics, although highly significant ($P = 0.025$ to $P < 0.0001$) variations in K_m and $k_{cat.}$ were detected. Against human α_1-proteinase inhibitor the K_i for MMCP-1C (45 pM) was significantly ($P < 0.0001$) greater than those for the other proteases (0.76–2.2 pM). The differences in electrophoretic mobility are probably a result of variable glycosylation, since removal of N-linked carbohydrate produced a polypeptide of approx. 28 kDa in each case which was, like the native enzyme, immunoreactive on Western blotting. A much less soluble 28 kDa enzyme was isolated from serosal mast cells and identified as MMCP-4 by N-terminal amino acid sequencing. Like MMCP-1 it has chymotrypsin-like substrate specificities with activity at neutral pH. However, it was antigenically distinct from MMCP-1 and, using sheep anti-MMCP-1, was not detected on Western blotting or by Ouchterlony double diffusion, e.l.i.s.a. or immunohistochemistry. This last technique established that the MMCP-1 variants were uniquely present in enteric mast cells, thereby providing a highly selective means of distinguishing the mucosal and connective tissue mast cell subsets in the mouse.

INTRODUCTION

Neutral serine proteases have been identified as major secretory granule components in the mast cells of several species, including the mouse (Du Buske et al., 1984; Newlands et al., 1987), rat (Lagunoff and Pritzl, 1976; Woodbury et al., 1978), dog (Caughey et al., 1987; Vanderslice et al., 1990) and man (Schwartz et al., 1981; Schechter et al., 1986). The distinctive, apparently tissue-specific, distribution of mast cell proteases in man and rodents suggests that different mast cell populations may respond according to the type of protease secreted.

Rat connective tissue mast cells (CTMC), typified by those found at serosal surfaces, contain the chymotrypsin-like enzyme rat mast cell protease I (RMCP I) (Lagunoff and Pritzl, 1976), while mucosal mast cells (MMC), found predominantly in the mucosa of the gastrointestinal tract, contain the similar, but antigenically distinct and more soluble, enzyme, RMCP II (Woodbury et al., 1978). Quantification of the distribution and of the systemic secretion of RMCP II during intestinal immunological reactions have been facilitated by the development of highly sensitive e.l.i.s.a.s (reviewed in Miller et al., 1990), and it is clear from these studies that RMCP II serves a very different function from that of RMCP I.

Mouse mast cell protease (MMCP-1), isolated from the intestinal mucosa of mice infected with the parasite *Trichinella spiralis*, shares 74 % amino acid sequence identity with RMCP II and also has a very similar tissue distribution and biochemical properties (Newlands et al., 1987; Miller et al., 1988; Le Trong et al., 1989; Huntley et al., 1990a). However, the protease content of murine mast cells is apparently more complex than that of the rat. Murine bone marrow-derived mast cells (BMMC), often regarded as *in vitro* analogues of MMC (Sredni et al., 1983), contain four mast cell proteases in the molecular mass range 28–33 kDa, including MMCP-1 as determined by Western blotting and e.l.i.s.a. (Newlands et al., 1991). Four esterases of similar or identical molecular masses which bind the specific serine protease inhibitor [^{3}H]di-isopropylfluorophosphate (DFP) have also been found in BMMC (Du Buske et al., 1984). Five proteases have been identified in CTMC and/or virus-immortalized mast cell lines on the basis of N-terminal amino acid sequences and molecular masses, and designated MMCP-2–MMCP-6 (Serafin et al., 1990; Reynolds et al., 1990). All but one (MMCP-3) of these proteases have been cloned and sequenced. MMCP-1 is expressed only in MMC and MMCP-2 is expressed in intestinal mucosa and Kirsten sarcoma virus-immortalized mast cells (KiSV-MC) (Serafin et al., 1991). MMCP-4 is transcribed in intestinal mucosa, CTMC and KiSV-MC (Serafin et al., 1991), and MMCP-5 in CTMC and KiSV-MC (Huang et al., 1991; McNeil et al., 1991). MMCP-6, identified as a tryptase by its predicted amino acid sequence, appears to be exclusive to CTMC and KiSV-MC (Reynolds et al., 1991).

The purpose of the present study was to define more fully the

Abbreviations used: α_1-PI, α_1-proteinase inhibitor; BMMC, bone marrow-derived mast cells; Cbz-L-Tyr-4NPE, carboxybenzoyl-L-tyrosine-4-nitrophenyl ester; CTMC, connective tissue mast cells; DFP, di-isopropylfluorophosphate; FITC, fluorescein isothiocyanate; HBSS, Hanks balanced salt solution; HRP, horseradish peroxidase; KiSV-MC, Kirsten sarcoma virus-immortalized mast cells; MMC, mucosal mast cells; MSMCP, mouse serosal mast cell protease; MMCP, mouse mast cell protease; PMSF, phenylmethanesulphonyl fluoride; RMCP, rat mast cell protease; Succ-Ala-Ala-Pro-Phe-4NA, succinyl-alanine-alanine-proline-phenylalanine-4-nitroanalide; t-PA, tissue plasminogen activator.

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MMCP-1-like proteases present in *Trichinella*-infected mouse intestine (Miller et al., 1990) and to compare their enzymic and antigenic properties with those of a novel, highly insoluble protease isolated from murine serosal mast cells.

MATERIALS AND METHODS

Male and female NIH mice, 8–10 weeks old, bred and maintained under conventional conditions at the Department of Zoology, University of Nottingham, U.K., were infected with 300 *T. spiralis* muscle larvae as described previously (Wakelin and Lloyd, 1976). Female Balb/c mice, 6 weeks old, were infected with 300 *Nippostrongylus brasiliensis* l_3 by subcutaneous injection. The mice were killed by cervical dislocation 10 days after infection. The small intestine of each mouse was removed and the lumen flushed through with Hanks balanced salt solution (HBSS) to remove digesta before storage at −20 °C or fixation in Carnoy's fluid and subsequent processing to paraffin wax for immunohistochemistry as described previously (Newlands et al., 1990). Porton mice, 6 months old, bred and reared at Moredun Research Institute, were used for isolation of murine serosal mast cell protease (MSMCP).

Isolation of enzymes

Murine intestinal mast cell proteases were isolated by a modification of the previously published method (Newlands et al., 1987). Briefly, after thawing, 10 g of small intestine was chopped finely with scissors and homogenized in 20 mM Tris/HCl, pH 7.5 (3 ml/g) with a Polytron homogenizer (Northern Media Supplies Ltd., North Cave, North Humberside, U.K.). The homogenate was centrifuged at 5000 *g* for 5 min and the resulting supernatant was centrifuged at 50000 *g* for 15 min. The final supernatant was applied to a 16 mm × 65 mm CM-Sepharose cation-exchange column (Pharmacia, Milton Keynes, Bucks., U.K.) equilibrated with 20 mM Tris/HCl, pH 7.5. Bound proteins were eluted with a 0–1.0 M NaCl gradient over 90 ml. Fractions (5 ml) were collected and screened for chymase activity against the synthetic substrate carboxybenzoyl-L-tyrosine 4-nitrophenyl ester (Cbz-L-Tyr-4NPE) (Sigma) as described previously (Knox et al., 1986). Those fractions which contained enzyme activity were pooled and applied to a 16 mm × 160 mm column of Sephadex G25 (Pharmacia) in order to rapidly desalt and exchange the buffer for 50 mM Mes (Sigma), pH 6.0. The desalted material was applied to a Mono-S cation-exchange column (Pharmacia) equilibrated with 50 mM Mes, pH 6.0, and eluted with a 50–150 mM NaCl gradient over 20 ml. Individual peaks were collected and those with activity against Cbz-L-Tyr-4NPE were diluted 5-fold with 50 mM Mes, pH 6.0, re-applied to Mono-S and again eluted with a 50–150 mM NaCl gradient.

Analysis of MSMCP

Mice were killed by cervical dislocation following anaesthesia with Halothane (May and Baker) and cells were recovered from the peritoneal cavity by lavage with 5 ml of HBSS containing 0.1 % (w/v) gelatin (Hanks/gelatin). Cells were sedimented by centrifugation (400 *g* for 10 min), washed once with Hanks/gelatin and resuspended in 1 ml of Hanks/gelatin. Mast cell numbers were evaluated using a haemocytometer after staining with 1 % (w/v) Methylene Blue in 50 % (v/v) propylene glycol. The cell suspension was again sedimented by centrifugation; the supernatant fluid was discarded and the cell pellet was stored at −20 °C.

A mast cell granule preparation was prepared by the method of Lagunoff and Pritzl (1976). Briefly, a thawed cell pellet was resuspended in 1 ml of distilled water and the cells were fully disrupted by sonication (MSE Sonicator) at 16 μm amplitude for 3 × 10 s with a 3 mm diameter probe. Granules were isolated from the lysate by a differential centrifugation technique, first at 200 *g* to remove nuclei and larger cell debris followed by centrifugation at 50000 *g* for 15 min to sediment the mast cell granules. The granule pellet was washed twice with PBS and finally solubilized in 1 ml of 2 M NaCl. The pellet extract was further centrifuged at 8000 *g* for 2 min in an Eppendorf bench-top centrifuge and the supernatant fluid was applied to a Sephacryl S-200 (Pharmacia) size exclusion column (10 mm × 200 mm) equilibrated with 1.0 M NaCl in 20 mM Tris/HCl, pH 7.5. Fractions (1 ml) were collected and tested for enzyme activity against Cbz-L-Tyr-4NPE. Those containing activity were pooled, diluted to a final concentration of 0.5 M NaCl with 20 mM Tris/HCl (pH 7.5) and applied to the Mono-S cation-exchange column equilibrated with 0.5 M NaCl in 20 mM Tris/HCl, pH 7.5. The column was eluted with a 0.5–1.0 M NaCl gradient over 10 ml. All centrifugation and extraction steps were carried out at 4 °C.

Protein estimations

Protein concentrations were estimated using a Pierce BCA protein assay kit (Pierce and Warriner, Cheshire, U.K.), using BSA as standard, in accordance with the manufacturer's instructions.

SDS/PAGE analysis

Discontinuous SDS/PAGE was carried out as described by Laemmli (1970) on 15 % mini slab gels (Mini Protein II; Bio-Rad) run at 200 V for 1.5 h, or on large format (Protean II xi; Bio-Rad) 10 % tricene/SDS/PAGE gels (Schagger and von Jagow, 1987) run overnight at 100 V (constant) to prepare samples for amino acid sequence analysis. A range of molecular mass standards was included in each run: lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), bovine carbonic anhydrase (30 kDa), ovalbumin (45 kDa), BSA (66.2 kDa) and phosphorylase B (97.4 kDa). Protein bands were visualized by staining with Coomassie Blue or by silver staining.

Electroblotting

Proteins in SDS/PAGE gels were transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) or poly(vinylidene difluoride) membranes (Immobilon P; Millipore) using a semi-dry transfer apparatus (Kyhse-Andersen, 1984) with a current of 0.1 mA/cm^2 of gel for 1 h. After transfer, nitrocellulose membranes were incubated in a solution of 0.1 % (w/v) Tween 20 (Sigma) in PBS, pH 7.5 (PBS/T20), for 30 min at room temperature to block non-specific protein adsorption to the membranes. Following the blocking procedure the membranes were rinsed in fresh PBS/T20 (all subsequent washes and antibody dilutions were in PBS/T20) and transferred to optimally diluted sheep anti-MMCP-1, rabbit anti-MMCP-1 or rabbit anti-RMCP I for 1 h at room temperature. The membranes were subsequently washed (3 × 5 min) and probed with optimally diluted pig anti-sheep IgG–horseradish peroxidase (HRP) conjugate prepared according to the method of Nakane and Kawaoi (1974), or with sheep anti-rabbit IgG–HRP conjugate (Sera, W. Crawley, Sussex, U.K). Peroxidase activity was revealed with diaminobenzidine/H_2O_2 (Graham and Karnovsky, 1966). Immobilon membranes with protein bands for sequence analysis were stained with Coomassie Blue immediately after transfer was completed, and destained in methanol.

Deglycosylation

Aliquots of the proteases (20–25 μl), containing approx. 2 μg of protein, were denatured by heating in a boiling-water bath for 10 min and then cooled to room temperature. Denatured samples were incubated with 10 μl of deglycosylation buffer [150 mM phosphate buffer, pH 7.0, 50 mM EDTA and 1% (v/v) 2-mercaptoethanol] and 1 μl (0.2 unit) of peptide-N-glycosidase F or endo-α-acetylgalactosaminidase (Boehringer–Mannheim, Lewes, E. Sussex, U.K.) at 37 °C overnight. An additional 0.2 unit of glycosidase was then added and incubation was continued for a further 24 h.

E.l.i.s.a.

MMCPs were quantified by an antibody capture e.l.i.s.a. described previously (Huntley et al., 1990a) except that the capture antibody was affinity-purified sheep anti-MMCP-1, which was diluted to 1 μg/ml for coating e.l.i.s.a. plates.

Immunohistochemistry

Tissue sections were stained with Toluidine Blue, pH 0.5 (Enerback, 1966), or with sheep anti-MMCP-1 directly conjugated to fluorescein isothiocyanate (FITC) (Rinderknecht, 1962), by the following method. Rehydrated sections (5 μm thick) were incubated in 5% (w/v) BSA (Sigma, Grade IV) in PBS for 30 min before transfer to sheep anti-MMCP-1–FITC, optimally diluted in 5% BSA/PBS for 1 h at room temperature. After washing (3 × 5 min in PBS), sections were mounted in Citifluor non-fluorescent mountant (Citifluor Ltd., London, U.K.).

Specific anti-protease antibodies

Rabbit antiserum to MMCP-1 was prepared as described previously (Newlands et al., 1987). Rabbit anti-RMCP I antibodies were raised, absorbed against MMCP-1-Sepharose and affinity purified on RCMP I–Sepharose as described previously (Miller et al., 1988). Antiserum to MMCP-1 was raised in sheep by intramuscular injection of 100 μg of MMCP-1 in Freund's complete adjuvant. Two subsequent injections of MMCP-1 in Freund's incomplete adjuvant were given 4 and 7 weeks after the first. The sheep was bled at 2-week intervals after the final injection and batches of serum with an antibody titre >1:16 by double diffusion against MMCP-1 were applied to an MMCP-1–Sepharose affinity column and eluted anti-MMCP-1 antibodies were cross-absorbed against RMCP I as described for rabbit anti-MMCP 1 (Miller et al., 1988).

Binding of [^{3}H]DFP

Protease samples (40 μl) containing 4–8 μg of protein were incubated with 10 μCi of [^{3}H]DFP (5.8 Ci/mmol; Amersham International plc) at 37 °C for 15 min. The samples were prepared for electrophoresis by the addition of 50 μl of reducing sample buffer (Laemmli, 1970) and heating to >90 °C for 3 min. After electrophoresis the gels were prepared for autoradiography by the method of Laskey and Mills (1975). Radiographs were exposed at −70 °C and developed after 21 days.

Substrate kinetics

All kinetic measurements were made using a Beckman DU 600 spectrophotometer. Rates of hydrolysis of five concentrations (0.125–2 mM) succinyl-alanine-alanine-proline-phenylalanine-4-nitroanalide (Succ-Ala-Ala-Pro-Phe-4NA) were measured by adding 2 μl of substrate solution to 48 μl of enzyme solution in 0.1 M Tris/HCl, pH 7.5. The increase in absorbance was continuously measured at 410 nm, and the spectrophotometer's on-board software was used to calculate the kinetic constants K_m and $k_{cat.}$ from the initial rates of substrate hydrolysis.

Concentrations of protease were determined by active-site titration of the MMCP-1-like proteases against the fluorogenic substrate 4-methylumbelliferyl-*p*-(*NNN*-trimethylammonium)-cinnamate (Sigma) (Jameson et al., 1973). Fluorescence was measured on a Perkin–Elmer 3000 fluorescence spectrophotometer with an excitation wavelength of 365 nm and an emission wavelength of 445 nm. Unknown samples were measured, in triplicate, against a standard curve prepared with 7-hydroxy-4-methylcoumarin (Aldrich Chemical Co. Ltd.).

Inhibitor sensitivity

The sensitivity of isolated enzymes to the following specific protease inhibitors was studied using Cbz-L-Tyr-4NPE as substrate: 2.0 mM phenylmethanesulphonyl fluoride (PMSF), 20 μM 3,4-dichloroisocoumarin, 200 μM *N*-[*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]-amido-(4-guanido)butane (E64), 2 mM 1,10-phenanthroline and 1 mM pepstatin (all reagents supplied by Sigma). The assay of enzyme activity was as follows. Samples of 2.5 μl of enzyme preparation and 2.5 μl of inhibitor were preincubated with 40 μl of 0.1 M Tris/HCl buffer, pH 7.5, at 22 °C for 1 h. The enzyme concentration in the incubation mixture was 50 nM and the inhibitor concentrations were those given above. After preincubation, 5 μl of substrate was added and incubated for a further 3 min. Enzyme activity was quenched by the addition of 450 μl of 70% (v/v) methanol. Absorbance was measured at 410 nm and the percentage inhibition was calculated in comparison with an enzyme preparation incubated without inhibitor.

The association constant ($k_{ass.}$) for the reaction between the MMCP-1-like proteases and human α_1-proteinase inhibitor (α_1-PI; Sigma) was determined by preincubating equimolar enzyme and α_1-PI for periods of 15–120 s before continuous measurement of the rate of hydrolysis of the substrate Succ-Ala-Ala-Pro-Phe-4NA. The $k_{ass.}$ for each reaction was calculated by non-linear regression as described previously (Pirie-Shepherd et al., 1991). The inhibition constant (K_i) for the reaction between each protease and α_1-PI was determined by incubating the protease with various inhibitor concentrations (0.25–1.75 molar equivalents) for $35 \times t_{\frac{1}{2}}$ (the time to half-inhibition for an equimolar reaction, calculated from the equation $t_{\frac{1}{2}} = 1/k_{ass.}E_0$, where E_0 is the initial enzyme concentration). The apparent K_i ($K_i^{app.}$) was calculated by non-linear regression, as described previously (Pirie-Shepherd et al., 1991), and the true K_i was calculated from the equation $K_i = K_i^{app.}/(1 + S_0/K_m)$, where S_0 is the initial substrate concentration.

Amino acid analysis

N-terminal sequence analyses of MMCP-1 A–E were performed on soluble proteins in 50 mM Mes directly from ion exchange on an Applied Biosystems 477A protein microsequencer by the Welmet Protein Characterization Facility, Biochemistry Department, University of Edinburgh. MSMCP, immobilized on Immobilon-P following tricene/SDS/PAGE and Western blotting, was sequenced in the Microchemical Facility, AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge, U.K.

RESULTS

Enzyme isolation

Enteric proteases

Cation-exchange chromatography of small intestinal homogenate in 20 mM Tris/HCl buffer, pH 7.5, on CM-Sepharose, when eluted with a linear 0–1.0 M NaCl gradient, gave one major peak which contained all the detectable enzyme activity against the synthetic substrate Cbz-L-Tyr-4NPE. After buffer exchange into 50 mM Mes, pH 6.0, the enzyme activity was resolved into five distinct peaks by f.p.l.c. cation-exchange chromatography on Mono-S (Figure 1). Each peak containing enzyme activity was collected separately, and after 5-fold dilution it was re-chromatographed on Mono-S, where each eluted at a different molarity (88, 92, 103, 109 and 115 mM NaCl for peaks 1–5 respectively). These fractions were labelled MMCP-1A to -1E, in order of elution, for identification.

Analysis of the peaks by SDS/PAGE under reducing conditions (Figure 2a) showed MMCP-1A to contain two polypeptides of 32 and 33 kDa of different staining intensities, which were not resolved by the techniques used. MMCP-1B and -1C each contained single proteins of 31 kDa, and MMCP-1D and -1E each contained a single band of 30 kDa.

MSMCP

Size-exclusion chromatography of the serosal mast cell granule extract on Sephacryl S-200 resolved two major peaks, the second of which contained enzyme activity which hydrolysed Cbz-L-Tyr-4NPE. When this material was applied to Mono-S cation exchanger, a single peak of bound material was eluted at 0.77 M NaCl. The protein resolved by cation-exchange chromatography hydrolysed Cbz-L-Tyr-4NPE and contained a single 28 kDa polypeptide on SDS/PAGE (Figure 2a).

Biochemical characterization

The MMCP-1 polypeptides in the 30–33 kDa range all bound the group-specific inhibitor [^{3}H]DFP (Figure 3), indicating the presence of serine esterase activity. The autoradiograph of the labelled proteins showed the same pattern as the silver-stained SDS/PAGE gel (Figure 2a), except that MMCP-1D had an additional polypeptide of 27 kDa labelled with [^{3}H]DFP which was not detectable on the stained gel (Figure 3). This additional poly-

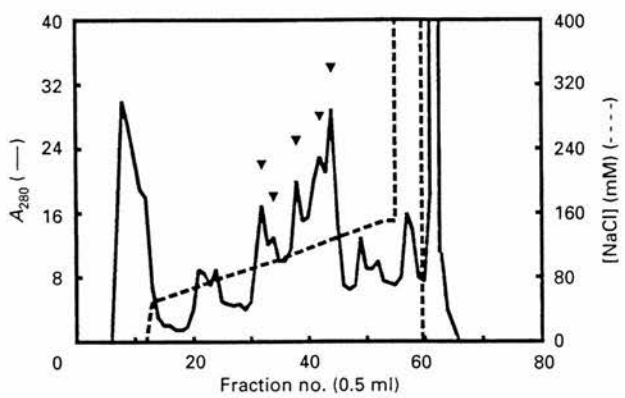

Figure 1 Mono-S cation-exchange chromatogram showing the resolution of five peaks of enzyme activity (arrowheads) against Cbz-L-Tyr-4NPE

The column was equilibrated with 50 mM Mes, pH 6.0, and eluted with a segmented gradient of 50–150 mM NaCl.

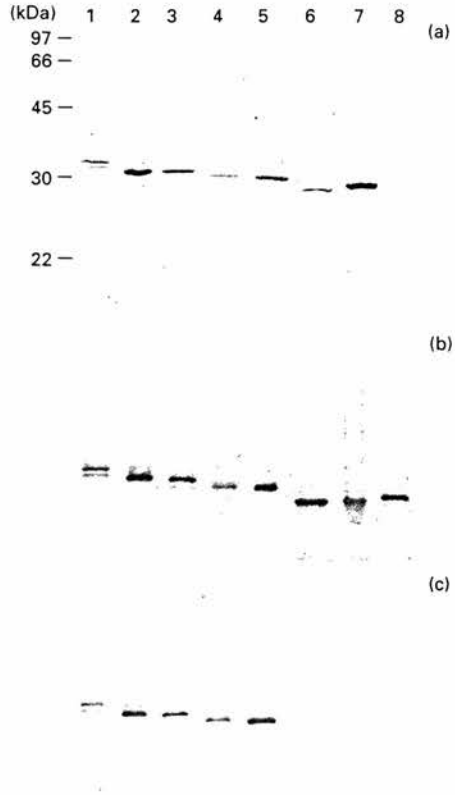

Figure 2 Analysis of MMCP-1 peaks

Characterization of MMCP-1 peaks from Mono-S cation-exchange on an SDS/15%-PAGE gel (silver staining). Peaks 1A–E from Mono-S were loaded in lanes 1–5 respectively. MSMCP is in lane 6, and RMCP I in lane 7. (**b**) and (**c**) Western blot analysis showing MMCP peaks 1A–E from Mono-S in lanes 1–5 respectively, MSMCP in lane 6, 10^4 purified mouse serosal mast cells in lane 7 and RMCP I in lane 8 probed with rabbit anti-MMCP-1 (**b**) or sheep anti-MMCP-1 absorbed to remove anti-RMCP I activity (**c**). Isolated enzymes were loaded at the rate of 40–60 ng per lane.

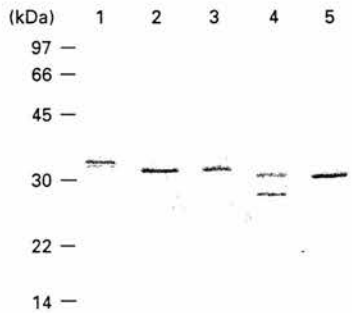

Figure 3 Autoradiograph of MMCP-1 peaks A–E labelled with the specific serine esterase inhibitor [^{3}H]DFP

Lanes 1–5 contain peaks A–E respectively.

Table 1 Substrate specificities of multiple forms of MMCP-1 and of MSMCP

Enzyme activity is expressed in nkat/mg of protein; 1 nkat is that activity which hydrolyses 1 nmol of substrate in 1 s. Bz, benzoyl; NA, no activity detected.

		Activity (nkat/mg)					
	Substrate	1A	1B	1C	1D	1E	MSMCP
Chymotryptic (esterase)	Cbz-L-Tyr-4NPE	63.0	42.0	31.0	33.0	21.0	259.0
	Cbz-L-Trp-4NPE	14.0	10.0	6.0	5.0	4.0	9.0
	Cbz-L-Phe-4NPE	0.3	0.9	NA	0.2	NA	NA
Elastinolytic (esterase)	Cbz-L-Ala-4NPE	15.0	6.0	4.0	9.0	4.0	41.0
	Cbz-D-Ala-4NPE	8.0	2.0	2.0	5.0	2.0	22.0
Typtic (esterase)	Cbz-L-Lys-4NPE	NA	NA	NA	NA	NA	NA
Chymotryptic (amide)	Bz-L-Tyr-4NA	1.0	NA	NA	NA	NA	2.0
	Succ-Phe-4NA	0.8	0.2	NA	NA	0.3	5.0
Tryptic (amide)	Cbz-L-Arg-4NA	2.0	NA	NA	0.4	NA	8.0
	Bz-DL-Arg-4NA	1.0	0.3	0.001	NA	NA	NA

Table 2 Substrate and inhibitor kinetics of the MMCP-1-like proteases

Conditions for substrate kinetics were 0.1 M Tris/HCl, pH 7.5, at 22 °C. Results were means ± S.E.M.

MMCP	K_m (μM)	$k_{cat.}$ (s^{-1})	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	$10^{-5} \times k_{ass.}$ ($M^{-1} \cdot s^{-1}$)	K_i (pM)
1A	922 ± 73.7	2.33 ± 0.10	2.5 ± 0.09	7.67 ± 1.50	0.76 ± 0.18
1B	991 ± 59.9	1.66 ± 0.04	1.7 ± 0.07	12.30 ± 0.35	1.02 ± 0.10
1C	663 ± 49.9	2.84 ± 0.12	4.3 ± 0.22	3.29 ± 0.48	45.40 ± 7.90
1D	936 ± 83.9	2.54 ± 0.12	2.7 ± 0.13	4.07 ± 0.62	2.20 ± 0.34
1E	832 ± 56.9	1.25 ± 0.04	1.5 ± 0.07	11.40 ± 0.22	1.51 ± 0.12
n	4	4	4	3	3
ANOVA	$P = 0.026$	$P < 0.0001$	$P < 0.0001$	$P < 0.001$	$P < 0.0001$

Table 3 Inhibition characteristics of mast cell proteases MMCP-1A–E and MSMC

Results are the percentage inhibition after 60 min of incubation with the inhibitor; $n = 3$ for each preparation.

	Inhibition (%)					
Inhibitor	1A	1B	1C	1D	1E	MSMC
PMSF	100	100	100	100	100	100
3,4-Dichloroisocoumarin	100	100	100	100	100	100
E64	5	18	5	9	0	0
1,10-Phenanthroline	8	5	11	5	0	0
Pepstatin	13	0	0	0	7	0

Table 4 MMCP concentrations and mast cell numbers in *N. brasiliensis*-infected and normal mice

Jejunal mast cell counts are expressed as cells per villus/crypt unit (vcu). *A, mast cells were abundant but were not counted.

	Control		Infected	
	Jejunum	Tongue	Jejunum	Tongue
MMCP (μg/g)	13 ± 3.6	0	183 ± 23.7	0.5 ± 0.12
Mast cell no. (Toluidine Blue)	1 ± 0.2	A*	7 ± 0.7	A*
Mast cell no. (fluorescence)	1 ± 0.3	0	6 ± 0.3	0

peptide may represent a degradation product generated during incubation with [^{3}H]DFP.

The five MMCP-1-like enzymes and MSMCP were screened against a range of low-molecular-mass synthetic protease substrates and were found to exhibit chymotrypsin-like esterase activity, as reported previously for MMCP-1 (Newlands et al., 1987); the results are summarized in Table 1. MSMCP also had chymotrypsin-like substrate specificities (Table 1). Using Succ-Ala-Ala-Pro-Phe-4NA as substrate, kinetic constants were calculated for each of the five MMCP-1-like enzymes. There were significant differences in K_m values ($P = 0.025$) with this substrate, and highly significant differences in both $k_{cat.}$ and $k_{cat.}/K_m$ ($P < 0.0001$ for each) by one-way analysis of variance. These results are summarized in Table 2.. The pH optima lay in the range pH 7.5–7.8 for all of the isolated enzymes (results not shown).

The five intestinal MMCPs and MSMCP were all completely inhibited by PMSF and 3,4-dichloroisocoumarin, both of which are inhibitors of serine proteases, but not by 1,10-phenanthroline, E64 or pepstatin, which inhibit metallo-, thiol and aspartic

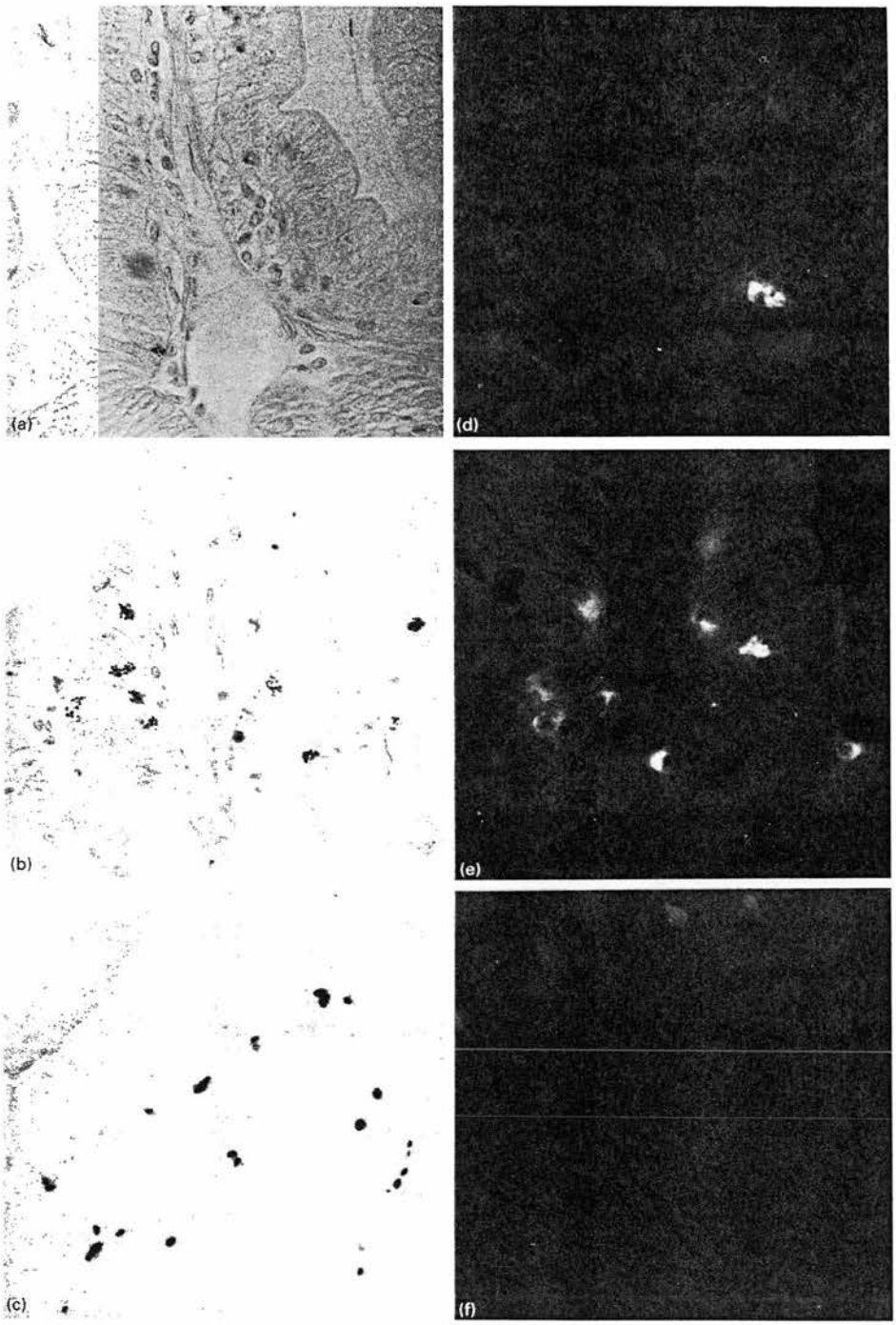

Figure 4 Photomicrographs showing normal mouse small intestine, *T. spiralis*-infected mouse small intestine and normal mouse tongue stained with Toluidine Blue, pH 0.5 (A–C respectively), or sheep anti-MMCP-1–FITC (D–F).

proteases respectively (Table 3). The MMCP-1-like proteases were also inhibited by human α_1-PI while MSMCP, like RMCP I in the rat (Pirie-Shepherd et al., 1991), was not. The association constants ($k_{ass.}$) for the reactions between the MMCP-1-like proteases and α_1-PI showed highly significant differences by one-way analysis of variance ($P = 0.001$). Inhibition constants obtained for the interaction of the MMCPs with α_1-PI showed K_is in the picomolar range, with the K_i of MMCP-1C being almost 60-fold greater than that of MMCP-1A ($P < 0.0001$; Table 2).

Immunological analysis

The isolated MMCPs were all identified by the rabbit antiserum raised against MMCP-1 (Newlands et al., 1987; Figure 2b) on Western blots, as was the 28 kDa MSMCP (Figure 2b). When a similar blot was probed with sheep anti-MMCP-1, cross-absorbed against RMCP I, only the highly soluble antigens isolated from small intestine were identified (Figure 2c).

The five MMCP-1-like enzymes shared complete lines of

	1	5	10	15	20	25	29
MMCP-1A	I I G G V E A R P H S R P Y M A H L K I I T D R G S E D R						
MMCP-1B	I I G G V E A R P H S R P Y M						
MMCP-1C	I I G G V E A R P H S R P Y M A H L K I I T D R G S E D R						
MMCP-1D	I I G G V E A R P H S R P Y M A H L K I I T D R G						
MMCP-1E	I I G G V E A R P H S R P Y M A H L K I I T D R G						
MSMCP	I I G G V E S R P H S R P Y M A H L E I						

Figure 5 N-terminal amino acid sequences of the variant glycoforms of MMCP-1 (A–E) and MSMCP (MMCP-4)

The single letter code is used to identify amino acids.

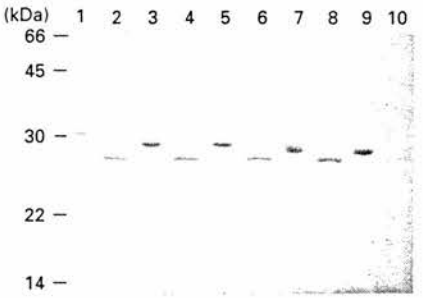

Figure 6 Silver-stained SDS/PAGE gel showing MMCP-1A to -1E before (lanes 1, 3, 5, 7 and 9 respectively) and after (lanes 2, 4, 6, 8 and 10 respectively) removal of N-linked carbohydrate with peptide-N-glycosidase F

identity in gel diffusion against sheep anti-MMCP-1, whereas no precipitation occurred with MSMCP (results not shown). When sheep anti-MMCP-1 was substituted with sheep anti-(RMCP I) a precipitin line was uniquely present against MSMCP and there was no reaction against MMCP-1 (results not shown).

All five MMCPs (0.5–12 ng/ml) were detected by e.l.i.s.a. using sheep anti-MMCP-1 as capture antibody, whereas MSMCP, RMCP I and RMCP II were not detected at 270 ng/ml, 2.5 μg/ml and 5.0 μg/ml respectively. After establishing the specificity of the e.l.i.s.a., samples of small intestine from *N. brasiliensis*-infected mice were assayed and MMCP-1 concentrations were found to be increased 14-fold when compared with uninfected control mice (Table 4). In contrast, tongues from control mice had no detectable MMCP-1 despite being rich in mast cells (Table 4; Figure 4). Small quantities of MMCP-1 were detected in tongues from infected mice (Table 4) and, as described previously (Huntley et al., 1990a), this is probably derived from the blood.

Immunohistochemical studies using the directly labelled sheep anti-MMCP-1–FITC conjugate confirmed the e.l.i.s.a. results, in that there were increased numbers of mast cells in the intestinal mucosa of parasitized mice which fluoresced after incubation with sheep anti-MMCP-1–FITC when compared with uninfected controls (Figure 4; Table 4). There were no mast cells detected by immunofluorescence in tongues of either control or parasitized mice despite the presence of abundant mast cells which stained with Toluidine Blue (Figure 4).

N-terminal sequencing

To determine whether the five intestinal mast cell proteases were separate gene products or differed because of post-translational processing, the N-terminal amino acid sequence was determined for each protease (Figure 5). The first 15–29 N-terminal residues of the intestinal mast cell proteases proved to be identical in each case and were in agreement with the previously published sequence for mouse intestinal mast cell protease (Le Trong et al., 1989). The amino acid sequence determined for MSMCP showed complete identity over the first 20 residues with that published for MMCP-4 (Reynolds et al., 1990).

Deglycosylation

To further investigate whether the differences between MMCP-1A–E are post-translational and a consequence of differential glycosylation, samples of each enzyme preparation were incubated with endo-α-acetylgalactosaminidase to remove O-linked carbohydrate, or with peptide-N-glycosidase F to remove N-linked carbohydrates. No change in the apparent molecular mass of any of the MMCPs, as determined by SDS/PAGE, was detected even after prolonged treatment with O-glycosidase. Following treatment of the five MMCPs with peptide-N-glycosidase F, however, there was degradation of each MMCP resulting in the appearance of a new polypeptide with an apparent molecular mass of 28 kDa (Figure 6). This 28 kDa polypeptide remained strongly antigenic on Western blot when probed with sheep anti-MMCP-1 (results not shown).

DISCUSSION

The five proteases isolated from *T. spiralis*-infected mouse small intestine can be identified as MMCP-1 by several criteria. Like MMCP-1, they are highly soluble, chymotrypsin-like proteases active at neutral pH. They are closely related antigenically both on Western blots and by Ouchterlony double diffusion, and are readily distinguished from MSMCP on the basis of their antigenicity. Finally, they share complete amino acid sequence identity over the first 15–29 N-terminal residues (Le Trong et al., 1989) which further distinguishes the MMCP 1 group of proteases from MMCPs 2–6 (Serafin et al., 1990; Reynolds et al., 1990), all of which differ from MMCP-1 within the first 10 N-terminal residues.

The differences in electrophoretic mobility between these variant forms of MMCP-1 are probably a result of variable glycosylation, since removal of N-linked carbohydrate moieties produced a new polypeptide of approx. 28 kDa from each of MMCP-1A–E which was antigenically similar to native MMCP-1. Analysis of the carbohydrate content of each enzyme preparation might prove helpful in further distinguishing between the different glycoforms of MMCP-1. It may be that each is produced by mast cells in different regions of the intestine or, more likely, by mast cells at varying stages of maturation, since intestinal nematodiasis induces massive mucosal mastocytosis with extensive recruitment and differentiation of intestinal mast cells (Miller et al., 1989).

Variable glycosylation occurs in other serine proteases, notably in the subunits of human mast cell tryptase (Cromlish et al., 1987) and tissue plasminogen activator (t-PA) (Wittwer et al., 1989). Human t-PA is synthesized in two forms, I and II, which share N-terminal amino acid sequences but have disparate carbohydrate side groups, the composition of which appears to be dependent on the cell line from which the t-PAs are derived (Wittwer et al., 1989). In the presence of a fibrinogen fragment stimulator, t-PA II will catabolize a synthetic substrate up to five times faster than does t-PA I (Wittwer et al., 1989), while type I is more resistant to cleavage by plasmin (Wittwer and Howard, 1990).

The kinetic data presented here show that there are significant differences in the rates at which the MMCP-1 proteases catalyse a low-molecular-mass substrate. This may be due to steric hindrance of substrate binding by the carbohydrate and suggests that glycosylation may be important in the catalysis of macromolecular protein substrates, possibly in aiding recognition of specific cleavage sites. The influence of glycosylation on the catalytic properties of MMCP-1 will not be fully understood until the native substrates are fully characterized.

The inhibition studies of the various glycoforms of MMCP-1 with human α_1-PI also clearly show that the catalytic properties of these enzymes are modified through their carbohydrate moieties, with highly significant variations in how rapidly they associate with inhibitor and how tightly they are bound. This may be important in prolonging enzyme activity in situations where immediate hypersensitivity reactions have increased vascular permeability, bathing surrounding tissues in serum containing high concentrations of serine protease inhibitors.

MSMCP, isolated from peritoneal CTMC, is, as far as we are aware, the first murine CTMC protease to be isolated in its native form and characterized biochemically. MSMCP is a neutral 28 kDa serine protease with chymotrypsin-like substrate specificities, properties it has in common with MMCP-1. It differs from MMCP-1 in that it is highly insoluble and, on cation-exchange chromatography, behaves like RMCP I which has a net charge of +18 (Le Trong et al., 1987). In contrast, MMCP-1 has similar chromatographic properties to RMCP II and has a net charge of +3 at neutral pH (Le Trong et al., 1989). N-terminal amino acid sequence analysis of MSMCP shows complete identity over the first 20 residues with the CTMC protease MMCP-4 (Reynolds et al., 1990; Serafin et al., 1991), differing at residues 7 and 19 from MMCP-1 A, C, D and E (MMCP-1B was not sequenced beyond residue 15). Based on this evidence, we can identify MSMCP as MMCP-4.

Immunological techniques have proved to be particularly useful in the analysis of protease distribution and function. For example, Woodbury et al. (1978) showed that RMCP I and II were readily distinguished by gel diffusion although they share 74% amino acid sequence identity. However, it was not possible to determine the cellular distribution of RMCP I and II without first preparing monospecific polyclonal antibodies by cross-absorption (Gibson and Miller, 1986) or by raising a monoclonal antibody against RMCP II (Huntley et al., 1990b). The distinction between the MMCP-1 family and MMCP-4 was, however, readily achieved with polyclonal sheep anti-MMCP-1 cross-absorbed against RMCP I and affinity purified on MMCP-1–Sepharose 4B. This was observed with gel diffusion, Western blotting, e.l.i.s.a. and immunohistochemistry. It was thus possible, using this antibody for immunohistochemistry, to confirm e.l.i.s.a. results (Huntley et al., 1990a) showing that the MMCP-1 family of proteases is not present in CTMC. As yet no antibodies are available which allow distinction between MMCP-1A–E, so it is not clear whether the different glycoforms are present in distinct regions of the small intestine or are being produced by MMC of varying maturity.

Using cDNA probes and Northern blotting, Serafin et al. (1991) detected expression of MMCP-4 in the intestines of parasitized mice. This may be in agreement with our findings of an RMCP I-like antigen expressed in mast cells in the gastrointestinal mucosae of *T. spiralis*-infected mice (Miller et al., 1988) although, in the rat, RMCP I is not present in intestinal mucosal mast cells (Huntley et al., 1990b). Serafin et al. (1991) were, however, unable to detect MMCP-4 in bone marrow-derived cultured mast cells by Northern blotting, although transcription of MMCP-4 has recently been reported in BMMC grown in medium containing the c-kit ligand growth factor (Gurish et al., 1992). An RMCP I-like antigen in murine BMMC, detected with anti-(RMCP I) by immunofluorescence and by immunoblotting of cell lysates, has a very similar or identical electrophoretic mobility to the mouse CTMC protease now identified as MMCP-4 (Newlands et al., 1991). It is therefore probable that MMCP-4 is present in BMMC grown in T-cell-conditioned medium, and the failure to detect it by Northern blotting may simply be due to the low levels of transcription. For example, murine BMMC contain < 120 ng of MMCP-1/10^6 cells (Newlands et al., 1991), compared with up to 34 μg of RMCP II/10^6 rat BMMC (Haig et al., 1988). These results again emphasize the potential value of highly specific antibodies in the analysis of protease distribution.

In summary, the five proteases isolated from mouse small intestine are identified as variant glycoforms of MMCP-1, and the novel CTMC protease is identified as MMCP-4. The biological significance of so many forms of MMCP-1 will only become clear when their native substrates and specific inhibitors are identified and their interactions characterized.

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Stem cell factor dependent hyperplasia of mucosal-type mast cells but not eosinophils in *Schistosoma mansoni*-infected rats

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SUMMARY

Stem cell factor (SCF) is a growth factor with multiple activities which acts on numerous cell types including primordial germ cells, haemopoietic stem cells, melanocytes and mast cells. SCF is critical for the development of the mast cell hyperplasia associated with infection with the intestinal parasites Nippostrongylus brasiliensis *and* Trichinella spiralis. *In the present study we have assessed the role of SCF in the mast cell and eosinophil responses to* Schistosoma mansoni *in the rat by blocking its effects* in vivo *with polyclonal antibody to SCF. Rats treated with sheep anti-SCF antibody on days 21, 24, 27 and 30 of infection with* S. mansoni *showed a rapid decrease in serum concentrations of the mucosal mast cell-associated protease rat mast cell protease II (RMCP II) by day 24, compared with normal sheep IgG-treated controls. Similarly, the number of mucosal mast cells and RMCP II levels in both small intestine and liver were also significantly reduced by day 32 of infection. In contrast with the depeletion of mast cells and mast cell proteases, eosinophil numbers in liver or intestine did not change significantly after anti-SCF treatment compared with controls. These results confirm that mast cell survival and hyperplasia are dependent on the presence of SCF whilst demonstrating that the eosinophil recruitment to liver and intestine associated with* S. mansoni *infection is SCF-independent.*

Keywords *stem cell factor, kit-ligand, mast cells,* S. mansoni

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INTRODUCTION

The cellular immune response to many helminth parasites is characterized by hyperplasia of mast cells and eosinophils both at the site of infection and systemically. Each of these cell types derive from haemopoietic progenitor lineages in bone marrow which differentiate and proliferate under the influence of an array of cytokines and growth factors. For mast cells these processes are regulated by interleukin (IL) 3, IL-4, IL-9 and IL-10 which are derived from T cells and possibly from other sources (Ihle, Pepersack & Rebar 1981, Schmitt *et al.* 1987, Hultner *et al.* 1990, Thompson-Snipes *et al.* 1991). In addition to this T cell-driven mechanism mast cell growth is regulated also by a ligand for c-*kit*, a tyrosine kinase receptor encoded for by the c-*kit* proto-oncogene, called stem cell factor (SCF) (Williams *et al.* 1990). SCF, produced by fibroblasts and other stromal cells, some epithelial cells and vascular endothelial cells, has multiple activities which regulate the development of c-kit^+ cell lineages including haemopoietic progenitor cells (Zsebo *et al.* 1990) and mast cells (Anderson *et al.* 1990) as well as primordial germ cells and melanocytes. IL-3, IL-5 and granulocyte-macrophage-colony stimulating factor (GM-CSF) all have a role in controlling proliferation and differentiation of eosinophil populations (reviewed by Sanderson 1991). Additionally, both IL-3 and IL-5 can act in synergy with SCF *in vitro* to give enhanced growth of eosinophils from an eosinophil precursor cell line (Kobayashi 1993). It is clear from this evidence that SCF has a role to play in generating the cellular immune response to parasites. Both mast cell and eosinophil numbers increase substantially in the liver and intestinal tract of rats, in response to infection with the intravascular parasite *Schistosoma mansoni* (Miller *et al.* 1994). Rat eosinophils, which mediate worm damage *in vitro* (Capron *et al.* 1981), may have a direct anti-parasite role. Furthermore, mast cells, which are

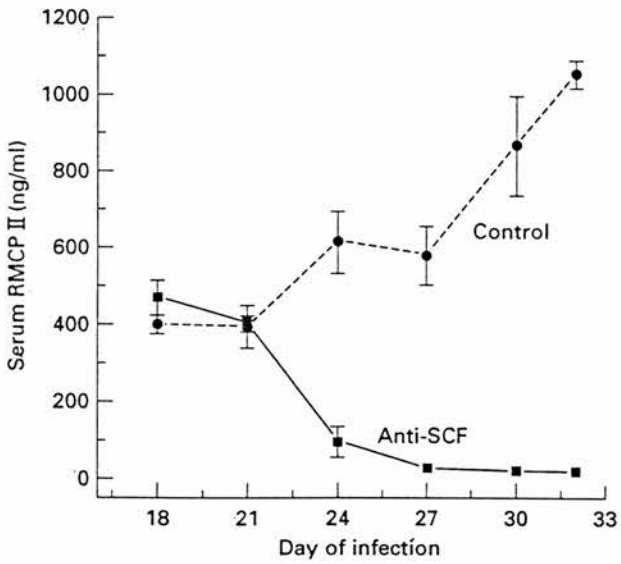

Figure 1 RMCP II concentrations in serum of *S. mansoni*-infected rats treated with anti-SCF or normal sheep IgG. The data presented are mean ± SEM for five animals per group at each time point. ANOVA: $P < 0{\cdot}0005$.

functionally active during the response to *S. mansoni*, (Miller *et al.* 1994), may have an accessory role. This may be mediated via anaphylactic release of a range of immune and inflammatory mediators through membrane bound, parasite-specific, IgE (Capron *et al.* 1980). The present study examined the role of SCF in mast cell and eosinophil hyperplasia by treating *S. mansoni*-infected rats with a polyclonal sheep anti-rat SCF antibody to determine whether SCF depletion altered mast cell or eosinophil numbers or concentrations of RMCP II in liver or small intestine.

MATERIALS AND METHODS

Fischer F344 inbred female rats, purchased from Harlan Olac (Bicester Oxon. UK), six to eight weeks old and weighing approximately 150 g, were used. They were maintained under standard conditions with water and pelleted diet freely available. Infection with 2500 *S. mansoni* cercariae via shaved abdominal skin was by the ring technique (Smithers & Terry 1965), carried out under anaesthesia with optimal doses of Hypnorm/Hypnovel administered by intra-peritoneal injection. Blood samples (approximately 300 μl per rat) were taken by tail-snip, under halothane anaesthesia, on days 18, 21, 24, 27, and 30 after infection. The blood was allowed to clot at room temperature and the serum was harvested and stored at −20°C. The rats were treated with 1 mg of affinity purified sheep anti-SCF antibodies or normal sheep IgG on days 21, 24, 27 and 30 by intraperitoneal injection. An interval of at least 2 h was allowed between blood-sampling and administration of antibody. On day 32 the rats were anaesthetized by intraperitoneal injection of Hypnorm/Hypnovel and blood samples taken as before. The hepatic portal system was perfused to recover adult worms (Smithers & Terry 1965). The liver was excised and a portion weighed prior to storage at −20°C. Slices cut from the remainder were fixed by immersion in 4% paraformaldehyde for 6 h and subsequently in 70% ethanol (Newlands *et al.* 1987). Similarly, a portion of jejunum was excised, flushed through with PBS and divided into two parts for storage at −20°C or fixation. Fixed tissues were processed and embedded in paraffin wax before sectioning and staining with either toluidine blue for the demonstration of mast cells or carbol chromotrope for eosinophils. Rat mast cell proteases I and II (RMCP I and II) were measured in serum and tissue extracts by antibody capture ELISA (Huntley *et al.* 1990). Cell counts were performed as described previously (Miller *et al.* 1994) and the results expressed as cells per 0·2 mm^2. Data are presented as mean ± SEM and were analysed by Student's two sample *t*-test or, for time course data by analysis of variance (ANOVA).

RESULTS

Serum concentrations of RMCP II increased in control rats from around 400 ng/ml on day 18 of infection with *S. mansoni* to 1045 ng/ml by day 32 (Figure 1); this accords with previously reported results (Miller *et al.* 1994). The concentrations of RMCP II in serum were significantly depressed ($P < 0{\cdot}0002$) in test rats, by day 24 of infection with *S. mansoni* (three days after the first injection of anti-SCF antibody) when compared with values in the controls treated with normal sheep IgG. The RMCP II concentrations in the blood of rats given anti-SCF antibodies decreased until only 12 ng/ml were detected on day 32 of infection, over 30-fold lower than values on day 21 and 87-fold lower when compared to controls (Figure 1).

Comparison of the time course data for serum RMCP II concentrations in treated and control rats showed a highly significant difference ($P < 0{\cdot}0005$ by ANOVA). Systemic concentrations of RMCP II in anti-SCF-treated and control rats were reflected by both the tissue protease concentrations, and mast cell counts, in liver and jejunum on day 32 of infection (summarized in Table 1); these values were reduced by >95% in each tissue. RMCP I, the connective tissue mast cell-associated protease, was not detectable in the jejunum

Table 1 Tissue mast cell protease concentrations and mast cell counts on day 32 of infection

	RMCP I	RMCP II	Mast cells	Eosinophils
Jejunum (anti-SCF)	0**	20·2 ± 6·2***	1·24 ± 0·37***	60·47 ± 5·44
Jejunum (control)	0·89 ± 0·128	524·0 ± 35·86	49·76 ± 3·15	66·35 ± 3·30
Liver (anti-SCF)	0·69 ± 0·14***	10·5 ± 2·48**	0·27 ± 0·11***	33·74 ± 5·92
Liver (control)	5·58 ± 0·62	260·0 ± 73·31	7·61 ± 1·02	44·56 ± 4·98

$n = 5$ for each group. Mast cell protease concentrations expressed in $\mu g/g$ wet wt. of tissue. Cell counts expressed as cells/0·2 mm^2. Student's two sample t-test, $^{**}P < 0.01$, $^{***}P < 0.0001$ comparing tissues from anti-SCF treated with control animals.

of anti-SCF treated rats whilst there was a >8-fold reduction($P < 0.0001$) in liver. Concentrations of RMCP II in the tissues similarly showed >25-fold lower values compared with controls (Table 1). This was in sharp contrast to the numbers of eosinophils in both liver and small intestine, which were not altered significantly by anti-SCF treatment (Table 1). Fewer worms were recovered by perfusion of the liver of treated rats (159 ± 10·7) than from control rats (189 ± 15·2), although the difference did not achieve statistical significance.

DISCUSSION

The present results provide additional evidence for the dependency on SCF of mast cell hyperplasia associated with parasitic infection while demonstrating that SCF is not a significant factor in the associated eosinophilia. A previous study showed that treatment of both normal and *N. brasiliensis* or *T. spiralis*-infected rats with polyclonal anti-SCF antibody significantly depressed mature mast cell numbers and jejunal and serum RMCP II concentrations, as well as parasite-associated mast cell hyperplasia (Newlands *et al.* 1995). Similarly, in the experiment described here, mast cell densities and serum protease concentrations were almost completely ablated. However, in contrast to the intestinal nematode infections, in which the mast cell numbers and protease values had begun to increase again by 6 days after the start of antibody treatment (Newlands *et al.* 1995), the mast cell numbers and serum RMCP II values in *S. mansoni*-infected rats remained depressed after 11 days of treatment with anti-SCF antibodies. The lack of any rebound response in mast cells may be due simply to a weaker antibody response to sheep IgG. It could indicate also that there is a different mechanism controlling mast cell hyperplasia in schistosomiasis than in the intestinal nematode infections, not involving T cell-derived cytokines such as IL-3.

Unaltered eosinophil numbers in the liver and jejunum of test and control rats suggest that eosinophil recruitment in parasitic infections is not significantly dependent on the presence of SCF or of mast cells, although SCF does synergise with IL-3 or IL-5 in promoting growth in an eosinophil cell line *in vitro* (Kobayashi *et al.* 1993). Similarly, when mice infected with *N. brasiliensis* were treated with antibody to IL-3, which suppressed mast cell hyperplasia, the associated eosinophilia in peripheral blood was undiminished (Madden *et al.* 1991) despite the fact that IL-3 is a recognized eosinophil growth factor. On the other hand, *S. mansoni* infected-mice, treated with monoclonal antibody to IL-5 showed complete ablation of the eosinophil response to schistosomula migrating through skin (Sher *et al.* 1990) indicating that IL-5 is the principal cytokine responsible for eosinophil production *in vivo*.

Expulsion of *T. spiralis* is substantially delayed in mast cell deficient, W/W^v mice (Alizadeh & Murrell 1984) and ablation of intestinal mast cells from mice by treatment with antibody to c-*kit*, the SCF receptor, abolished parasite expulsion (Grencis *et al.* 1993) confirming the mast cell dependent nature of the expulsion mechanism for *T. spiralis*. In contrast, *N. brasiliensis* was expelled normally from mast cell deficient, Ws/Ws rats (Arizono *et al.* 1993) and parasite fecundity was reduced in both Ws/Ws rats (Arizono *et al.* 1993) and anti-SCF treated rats (Newlands *et al.* 1995). Additionally suppression of the IgE response with anti-IgE antibodies decreased both the worm burden and egg production associated with *S. mansoni* infection in mice (Amiri *et al.* 1994). These data suggest that, whilst mast cells are probably an essential component of the expulsion mechanism of *T. spiralis* they are less important in the immune mechanisms directed against *N. brasiliensis* and *S. mansoni*, despite the fact that the mast cells are functionally active as evidenced by the systemic secretion of mast cell proteases (Miller *et al.* 1983, 1994). Indeed these data, coupled with the decrease in the number of parasites recovered from mast cell depleted rats reported here (not statistically significant) suggest that in some

parasitic infections, worm survival and fecundity may be enhanced by some aspect of the mast cell-IgE dependent immune responses.

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Stem Cell Factor Contributes to Intestinal Mucosal Mast Cell Hyperplasia in Rats Infected With *Nippostrongylus brasiliensis* or *Trichinella spiralis*, but Anti-Stem Cell Factor Treatment Decreases Parasite Egg Production During *N brasiliensis* Infection

By George F.J. Newlands, Hugh R.P. Miller, Anne MacKellar, and Stephen J. Galli

We assessed the effects of the c-*kit* ligand, stem cell factor (SCF), in the jejunal mucosal mast cell hyperplasia that occurs during infection with the intestinal nematodes, *Nippostrongylus brasiliensis* or *Trichinella spiralis* in rats. Compared with vehicle-treated rats, rats treated with SCF (25 μg/kg/d, intravenous [IV] for 14 days) during *N brasiliensis* infection exhibited significantly higher levels of the rat mucosal mast cell (MMC)-associated protease, rat mast cell protease II (RMCP II) in the jejunum and serum on day 8 of infection, but not on days 10 or 15 of infection. By contrast, in comparison to rats treated with normal sheep IgG, rats treated with a polyclonal sheep antirat SCF antibody exhibited markedly decreased numbers of jejunal MMCs, levels of jejunal RMCP II, and serum concentrations of RMCP II during infection with either nematode, particularly at the earlier intervals of infection (≤day 10). Taken together, these findings indicate that SCF importantly contributes to MMC hyperplasia and/or survival during *N brasiliensis* or *T spiralis* infection in rats, but that levels of endogenous SCF are adequate to sustain near maximal MMC hyperplasia during infection with these nematodes. Notably, treatment of rats with SCF somewhat increased, and treatment with anti-SCF significantly decreased, parasite egg production during *N brasiliensis* infection. This finding raises the interesting possibility that certain activities of intestinal MMCs may contribute to parasite fecundity during infection with this nematode.

AT LEAST TWO distinct mechanisms regulate mast cell proliferation, differentiation, and survival in murine rodents. One of these is mediated by a ligand for c-*kit*, which is a tyrosine kinase receptor that is encoded by the c-*kit* protooncogene.[1-4] The c-*kit* ligand is a growth factor that is produced by many cells, including fibroblasts and other stromal cells, some epithelial cells, and vascular endothelial cells; this growth factor has multiple activities that influence the development of c-*kit*+ lineages, including hematopoietic progenitor cells and mast cells, as well as primordial germ cells, melanocytes, and certain other cell types.[1-3] The c-*kit* ligand has been named Steel factor, *kit*-ligand, mast cell growth factor and stem cell factor (SCF), the designation that will be used herein.[1-3] Several lines of evidence indicate that, under physiological conditions in vivo, SCF is critical for the development and survival of all mouse and rat mast cells, including connective tissue-type mast cells, such as serosal mast cells, and intestinal mucosal mast cells (MMCs).[3,4] The other mechanism that regulates mast cell development in mice and rats is mediated by T cells and possibly other sources of interleukin-3 (IL-3), IL-4, IL-9, and IL-10, cytokines that can promote and/or copromote the proliferation and/or survival of certain murine mast cell populations, including the MMC-like bone marrow-derived mast cells that can be generated in vitro.[3-6]

In murine rodents, infection with intestinal nematodes induces a striking hyperplasia of intestinal MMCs, which is accompanied by increases in the levels of MMC-associated proteases in the intestinal tissues and increases in the concentrations of these proteases in the blood.[7-10] Previous work indicates that both SCF– and T-cell–dependent mechanisms contribute to the intestinal MMC hyperplasia associated with nematode infection. In comparison to normal mice or rats, mice or rats with mutations that markedly diminish c-*kit* receptor tyrosine kinase activity, such as W/W^v mice[11] or *Ws*/*Ws* rats,[12] exhibit no or greatly diminished hyperplasia of intestinal MMCs in response to infection with *N brasiliensis*[11,12] or *T spiralis*.[11] Both intestinal MMC hyperplasia and the spontaneous expulsion of the parasites are diminished in *T spiralis*–infected mice that have been treated with an antibody to the c-*kit* receptor.[13] Moreover, treatment of normal rats with *Escherichia coli*-derived recombinant rat SCF[164] which represents virtually the entire extracellular ligand domain of SCF and possesses high biologic activity,[3] induces a significant hyperplasia of gastrointestinal MMCs, as well as connective tissue-type mast cells.[14] On the other hand, athymic "nude" mice fail to develop MMC hyperplasia in response to nematode infection, whereas this response is restored in nude animals treated by adoptive transfer of T cells.[11] Moreover, in normal mice, treatment with either anti–IL-3 or anti–IL-4 antibodies or, even more effectively, treatment with both antibodies, significantly suppresses intestinal MMC hyperplasia in response to *N brasiliensis* infection.[15] Finally, in vitro evidence indicates that SCF and IL-3 can have synergistic effects in promoting the proliferation of rat mast cells with phenotypic similarities to MMCs.[16,17]

Taken together, this evidence indicates that the cytokine-dependent regulation of MMC hyperplasia during nematode infection in murine rodents, while quite complex, impor-

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tantly involves both SCF– and T-cell–derived cytokines, such as IL-3 and IL-4. In the present study, we assessed whether the administration of exogenous rrSCF[164] in amounts sufficient to induce MMC hyperplasia in normal (uninfected) rats, influenced either the jejunal MMC hyperplasia or the changes in levels of jejunal or serum MMC-associated RMCP II in rats infected with *N brasiliensis.* We also assessed whether treatment of normal or nematode-infected rats with a polyclonal sheep antibody to rat SCF altered MMC numbers, RMCP II levels, or, in *N brasiliensis*–infected rats, parasite fecundity.

MATERIALS AND METHODS

Rats

Random-bred female Wistar rats, weighing 250 to 300 g and maintained under conventional conditions at Moredun Research Institute, were used in all studies.

Parasites

Rats were infected with 2,500 to 3,000 *N brasiliensis* third-stage larvae by subcutaneous injection[9] or with 750 to 1,000 *T spiralis* muscle larvae per os.[10] For both of these procedures, the rats were anesthetized with Halothane (May and Baker, Dagenham, Essex, UK).

Stem Cell Factor

Recombinant rat stem cell factor[164] (rrSCF,[164] Amgen, Inc., Thousand Oaks, CA) modified by the covalent attachment of polyethylene glycol[14] was administered intravenously to anesthetized rats, at 25 μg/kg of body weight/day in 1 mL physiological saline, for 14 consecutive days; control rats were sham treated with physiological saline alone. The protocols were the same as those described previously.[14]

Antibodies

Polyclonal anti-rrSCF[164] antibodies were produced by inoculating sheep intramuscularly into both hind legs with 100 μg of rrSCF,[164] suspended in 4 mL of Complete Freund's Adjuvant. This procedure was repeated 4 and 8 weeks after the initial injection, except that Incomplete Freund's Adjuvant was used for the subsequent inoculations. Blood samples were taken 3 weeks after the final inoculation and the serum tested by enzyme-linked immunosorbent assay (ELISA) for anti-rrSCF[164] activity. Immune sera that gave absorbances more than fivefold greater than the background absorbance of preimmunization serum when tested at dilutions of up to 1/80,000 by ELISA were used to prepare specific antibody. The anti-SCF antibody preparation virtually abolished the effects of rrSCF[164] on the survival and proliferation of in vitro-derived rat mast cells, but had no detectable effect on the IL-3–dependent proliferation of these cells (submitted for publication).

An immuno-affinity column was prepared by coupling 1.6 mg of rrSCF to CNBr-activated Sepharose-4B (Pharmacia Biotech, St Albans, Herts, UK) in accordance with the manufacturer's instructions. Aliquots of serum (5 mL) were applied to the column, which had been equilibrated with phosphate buffered saline (PBS), and the column was subsequently eluted with 0.1 mol/L citric acid pH 2.2 + 0.5 mol/L NaCl to recover bound specific antibody. For control purposes, sheep immunoglobulin was prepared by precipitation of normal sheep serum with 50% saturated ammonium sulphate followed by gel filtration chromatography of the redissolved precipitate on Sephacryl S-200 (Pharmacia Biotech). Immunoglobulin preparations were concentrated by vacuum dialysis in a collodion thimble apparatus (Sartorious, Epsom, Surrey, UK) against PBS.

Treatment With Anti-SCF Antibodies

Rats were inoculated intraperitoneally with 1 mg sheep anti-rrSCF[164] or normal sheep IgG in 1 mL PBS. Normal rats were treated daily for each of 4 or 7 days to assess the effects of treatment on resting mast cell populations. Because of the tissue and pulmonary migration of the L_3 and L_4 larval stages of *N brasiliensis,* rats infected with this parasite were treated on day 3 of infection when the L_5 larvae first reached the intestine and then on days 5, 7, 10, and 12. A further experiment was performed with *N brasiliensis*–infected rats to determine whether treatment with anti-SCF had an effect on mast cell populations already expanded in response to parasitic infection. In this experiment, rats were treated with anti-SCF daily on days 10 to 13 after infection. For rats infected with *T spiralis,* treatment commenced on day 0 and continued on days 3, 5, 7, and 10. Normal rats were killed 24 hours after, and parasitized rats 24 or 48 hours after, the final inoculation by exsanguination under deep Halothane anesthesia followed by cervical dislocation.

Hematologic Studies

Blood samples were obtained by tail-snip under halothane anesthesia with the blood collected into heparinized tubes. Total red blood cell and leukocyte counts were performed on a model ZM Coulter counter, hemoglobin concentrations were measured on a Coulter hemoglobinometer and packed cell volume by micohematocrit. Blood films were stained with Leishman's stain for differential cell counts in which 100 leukocytes were counted per blood film.

Material Collected Postmortem

Peritoneal cells were collected by lavage of the peritoneal cavity with 20 mL of PBS containing 0.1% wt/vol bovine serum albumin (BSA) (PBS/BSA). Small intestine (mid-jejunum) was also collected for analysis and the tissues were either stored frozen at −20°C, or fixed by immersion in 4% wt/vol paraformaldehyde in PBS for 6 hours followed by 70% vol/vol ethanol overnight 14 before processing to paraffin wax, before further processing. Serum was also stored at −20°C. Peritoneal lavage samples were sedimented by centrifugation at 1,000*g* for 20 minutes at +4°C. The cells were resuspended in 1 mL PBS/BSA, recentrifuged at 1,000*g* for 5 minutes and finally resuspended in 1 mL PBS/BSA for estimation of mast cell numbers. Mast cell counts were performed by diluting a 10-μL aliquot of the cell suspension with 90 μL of a mast cell stain containing 0.5% wt/vol methylene blue in 50% vol/vol propylene glycol. Blue staining mast cells were counted using an Improved Neubauer hemocytometer.

Concentrations of Rat Mast Cell Proteases I or II (RMCP I or II) were measured in serum and tissue homogenates by ELISA as described previously.[8]

Assessment of Rat Antisheep Antibodies

Because ruminant proteins are likely to have been included in the rats' diet, and the experimental rats may have mounted an immune response against sheep proteins, an ELISA was developed to quantify the antibody response against sheep immunoglobulins. ELISA plates (Dynatech M129B) were coated with a solution of normal sheep immunoglobulin, prepared as described above, in 0.1 mol carbonate buffer pH 9.6 (50 μL/well). The plate was incubated at +4°C overnight and then washed six times with PBS containing 0.05% vol/vol Tween 20 (Sigma, Poole, Dorset, UK) before loading with samples. Sera from the animals that had been treated with anti-rrSCF[164] or normal sheep IgG, and pretreatment serum samples from

Table 1. Effects of SCF Treatment on Jejunal Mast Cell Density and Mast Cell Protease Content in Rats Infected With *N brasiliensis*

			Day of Infection		
	SCF	Control* (not infected)	8	10	15
Mast cells/0.2 mm^2		n = 5	n = 3	n = 5	n = 7
	−	26 ± 1.2	0.0 ± 0†	72 ± 16†	238 ± 27‡
	+	49 ± 4.2§	13 ± 6.8‖	69 ± 24	221 ± 40‡
RMCP I (μg/g)	−	3.0 ± 0.9	1.0 ± 0.4	1.0 ± 0.2	7.0 ± 2.3
	+	6.0 ± 1.5	2.0 ± 0.4	4.0 ± 0.9	20 ± 5.8
RMCP II (μg/g)	−	426 ± 59	19 ± 4.7‖	423 ± 181	2,464 ± 607†
	+	470 ± 60	375 ± 79¶	776 ± 281	8,711 ± 3,839

* These rats were treated with SCF or vehicle for 14 days and were killed for analysis of jejunal histology and RMCP I and RMCP II content 1 day later.

† $P < .02$ *v* values for corresponding control (not infected) group.

‡ $P < .001$.

§ $P < .01$.

‖ $P < .005$.

¶ $P < .05$ *v* values for SCF untreated group at the same time point.

the same rats, were prepared in serial dilutions in PBS and loaded, in triplicate, onto the plate (50 μL/well) and then incubated at room temperature for 1 hour. The ELISA plate was washed six times with PBS/Tween 20 and a sheep antirat IgG-horseradish peroxidase conjugate (Sigma), optimally diluted with PBS/Tween 20, was applied. The plate was again incubated at room temperature for 1 hour, washed six times as before and the color reaction developed, using orthophenylenediamine (0.4 mg/mL in citrate/phosphate buffer pH 5.0, 50 μL/well) as substrate, for 10 minutes and the reaction was terminated by the addition of 25 μL of 2.5 mol H_2SO_4. The plate was read on a Titertek Multiskan MC ELISA plate reader (Titertek, Paisley, UK) at 492 nm. Samples that had absorbances, which were significantly higher by Student's *t*-test than the pretreatment control samples, were considered to be positive.

Histological sections (5 μm thick) were stained with Toluidine blue (0.5% wt/vol, pH 0.5) and mast cells in the jejunal mucosa (epithelium and lamina propria) were enumerated on a Leitz Dialux microscope with a ×25 objective lens and ×12.5 eyepieces equipped with a 100 mm^2 graticule. A minimum of five fields were counted per section.

Data Presentation and Statistical Analysis

Unless otherwise specified, all data are presented as the mean ± SEM. The two-tailed Student's *t*-test was used to analyze data that were normally distributed, whereas the Mann-Whitney U-test was used as the nonparametric test. The time course of responses in different groups of rats were examined for statistical significance by using analysis of variance (ANOVA). $P < .05$ was taken to indicate a significant difference.

RESULTS

Effects of rrSCF^{164} Treatment on Jejunal MMC Numbers and Mast Cell-Associated Proteases in N brasiliensis*–Infected Rats*

Infection of rats with *N brasiliensis* causes an initial depletion of MMC from the jejunum, followed by a massive MMC hyperplasia.[7] The data in Table 1 confirm that mast cells were depleted from the jejunum at day 8 of infection (0 ± 0 MMC/0.2 mm^2; n = 3) compared with control values in noninfected rats (26 ± 1.2 MMC/0.2 mm^2; n = 5, $P < .02$; Table 1). By day 15 of infection, mast cell densities in the jejunum of vehicle-treated rats were increased ninefold over control values ($P < .0001$; Table 1). The concentrations of RMCP II reflected the MMC counts in the jejunum, with a 5.8-fold increase on day 15 when compared with control values in rats not treated with rrSCF^{164} ($P < .02$; Table 1).

Treatment of parasitised rats with rrSCF,^{164} beginning on day 0, resulted in a significant (~85%) elevation of mean jejunal mast cell density at baseline, a result that is in good agreement with our previous report.[14] However, the mast cell densities at 8, 10, or 15 days of infection in the rrSCF^{164}-treated or -untreated rats were not significantly different. There were more mast cells at day 8 in the specimens from rrSCF^{164}- versus -untreated rats, but with a group size of 3, this difference was not significant. On the other hand, the ~20-fold difference in jejunal RMCP II content on day 8 between rrSCF^{164}-treated and -untreated parasitized rats was significant ($P < .05$; Table 1).

Data for RMCP I content confirm previous findings showing that the concentration of RMCP I in the rat jejunum is several orders of magnitudes less than that of RMCP II,[9] and these values changed to a lesser extent than values for RMCP II as a result of *N brasiliensis* infection (Table 1).

Effects of rrSCF^{164} Treatment on Systemic Secretion of RMCP II During Infection

Daily intravenous injection of rrSCF^{164} had no effect on the systemic secretion of RMCP II in control (uninfected) rats, in which serum values remained at ≅300 ng/mL in both SCF-treated and -untreated controls throughout the course of the experiment (Fig 1). The concentration of RMCP II increased to 2350 ng/mL in the control parasitised group by day 10 of infection, but the peak of serum RMCP II values occurred earlier, on day 8, in the rrSCF^{164}-treated group. The day 8 values in the SCF-treated infected group (individual values = 3,250, 4,250 and 6,450 ng/mL, mean ± SEM = 4,650 ± 945 ng/mL) were substantially higher than the corresponding values in the vehicle-treated infected rats (individual values = 1,200, 1,650, and 2,150 ng/mL, mean ± SEM = 1,670 ± 274 ng/mL). While the number of observations

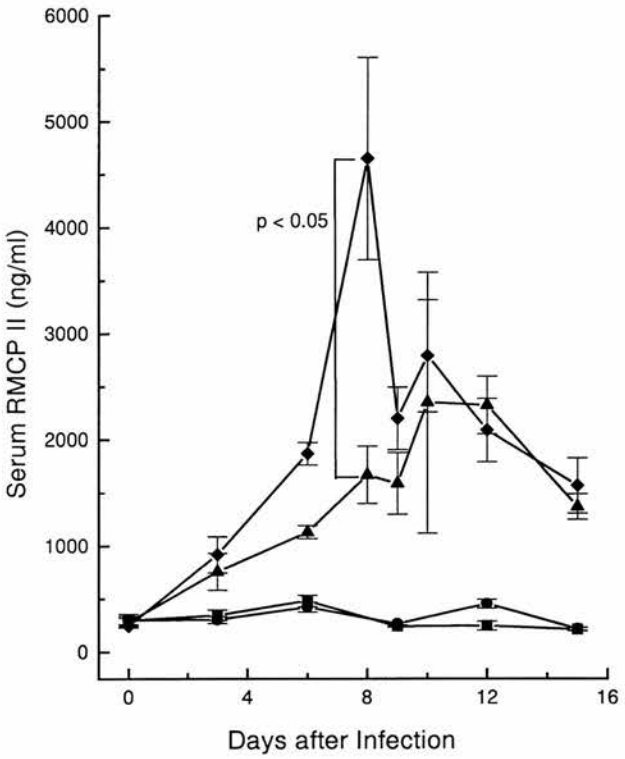

Fig 1. Serum concentrations of RMCP II in uninfected rats treated with rrSCF^{164} (●) or vehicle alone (■) compared with *N brasiliensis*–infected animals treated with rrSCF^{164} (♦) or vehicle controls (▲) (n = 4 to 8 for each interval in each group except for the day 8 values; n = 3 for this interval in both groups).

was relatively small at this time point (n = 3/group), there were no overlapping values in the two groups, and the difference between the groups was significant at the $P < .05$ level. Together with the data in Table 1, this result shows that, at day 8 of infection, rrSCF^{164} treatment results in both increased jejunal RMCP II content and increased secretion of this mast cell protease. Moreover, by ANOVA, serum levels of RMCP II over the entire time course of the infection were significantly higher in the rrSCF^{164}-treated than in the control group not treated with the cytokine ($P = .001$).

Effects of Treatment of Rats With Anti-SCF

Development of an antibody response to sheep Igs in rats treated with the polyclonal sheep anti-SCF antibodies. On day 6 of treatment with either sheep anti-SCF or normal sheep IgG, *T spiralis*–infected rats had an antisheep Ig antibody titre of 320, as assessed by ELISA, when compared with pretreatment sera from the same group of rats ($P < .05$). Interestingly, although the assay of sera from day 12 of treatment gave somewhat higher absorbances than that from the day 6 groups, the titre remained at 320 ($P < .05$). To determine whether this was a genuine antisheep IgG response or simply a reflection of upregulated IgG production as a result of infection, the sera from *N brasiliensis*–infected rats that had not been treated with sheep Igs were also assayed. No significant difference in absorbance was found on days 3, 6, 9, or 12 of infection when compared with serum samples obtained from the same group of rats before infection.

Effects of treatment with anti-SCF in normal (uninfected) rats. When compared with rats treated with normal sheep IgG, 4 days of treatment with polyclonal sheep anti-rrSCF depleted serosal mast cells in the peritoneal cavity by greater than 56% ($P < .05$) and decreased the concentration of the major serosal mast cell-associated protease (RMCP I) in the cell pellet obtained at peritoneal lavage by 65% (Table 2). These levels of depletion did not increase by prolonging the treatment with anti-rrSCF for 7 days (data not shown).

No mast cells at all were detected after anti-SCF treatment in sections of jejunum ($P < .001$ *v* values for control rats) and both RMCP I and RMCP II concentrations were depleted at this site ($P < .05$ or $P < .0001$, respectively, Table 2). The depression of jejunal mast cell densities and RMCP II concentrations were reflected in the systemic levels of RMCP II, which were depleted by 96% in the serum of anti-SCF-treated rats (Table 2).

Anti-SCF treatment for 4 days also resulted in a 45% reduction in peripheral blood white blood cells, as assessed in rats killed for hematological analysis on day 5 ($P < .02$) (Table 3). From the differential cell counts, this appeared to represent an effect on all types of leukocytes (Table 3). The proportion of neutrophils counted in both the anti-SCF and normal IgG-treated control groups was depressed compared with pretreatment values (Table 3), possibly as a result of the stress induced by daily handling for inoculation and blood sampling and/or in response to the foreign protein.

Infection with N brasiliensis. *N brasiliensis*–infected rats were first treated with anti-SCF or normal sheep IgG on day 3 of infection when the worms begin to emerge into the gastrointestinal tract. As expected, mast cell counts in both control and treated groups were low on day 6 of infection (Fig 2). This result is in accord with previous work indicating that *N brasiliensis* infection produces an initial reduction in

Table 2. Effects of Anti-SCF Treatment for Four Days on Mast Cell Populations in Normal Rats

	Anti-SCF	Mast Cells/ 0.2 mm^2	RMCP I (μg/g)*	RMCP II (μg/g)
Jejunum	–	6.5 ± 1.4	0.84 ± 0.22	343 ± 45
	+	0.0 ± 0†	0.24 ± 0.08‡	0.4 ± 0.25§
Peritoneal mast cells ($\times 10^5$/ lavage)	–	5.1 ± 1.0	98 ± 8.6	ND
	+	2.2 ± 0.6‡	34 ± 15‡	ND
Serum (ng/mL)	–	NA	NA	157 ± 15
	+	NA	NA	7.0 ± 1.9§

Each of the two groups (+ or – anti-SCF treatment) contained five rats that were killed 24 hours after the last injection of anti-SCF or normal sheep IgG.

Abbreviations: NA, not applicable; ND, not determined.

* RMCP I was measured per lavage in peritoneal mast cells, and is expressed for peritoneal mast cells only as μg/lavage.

† $P < .001$ by two-tailed Student's *t*-test *v* values for anti-SCF–untreated animals.

‡ $P < .05$.

§ $P < .0001$.

Table 3. Hematologic Parameters of Rats Treated for Four Days With Sheep Anti-SCF (anti-SCF) or With Normal Sheep IgG (Control) Compared With Pretreatment (Day 0) Controls

	WBC ($\times 10^{10}$/L)	RBC ($\times 10^{12}$/L)	Hb (g/dL)	Hct (%)	Differential Count (%)			
					Neutrophils	Lymphocytes	Monocytes	Eosinophils
Day 0	1.1 ± 0.05	6.7 ± 0.1	15.4 ± 0.3	40.5 ± 0.5	14.9 ± 1.5	78 ± 2.0	6.1 ± 0.7	1.1 ± 0.4
anti-SCF Day 5	0.6 ± 0.04*	6.3 ± 0.2	14.6 ± 0.6	39.4 ± 1.1	5.0 ± 1.1	85 ± 2.5	7.8 ± 1.9	1.8 ± 0.7
control Day 5	1.1 ± 0.16	6.1 ± 0.4	14.9 ± 0.8	38.8 ± 1.6	5.2 ± 1.6	88 ± 1.8	5.0 ± 1.4	1.8 ± 0.7

Each of the groups contained five rats that were killed 24 hours after the last injection of anti-SCF or normal sheep IgG. Thus, n = 10 for day 0 values (after blood was drawn on day 0, 5 of the 10 rats received anti-SCF treatment, and the other 5 received normal sheep IgG treatment), whereas n = 5 for the day 5 values.

* $P < .02$ by two-tailed Student's *t*-test *v* day 5 control.

mast cell densities in the intestines[16] and other, distant, sites.[17] However anti-SCF treatment resulted in significant inhibition of mast cell hyperplasia (Fig 2A). This effect was most notable on day 10 of infection ($P < .001$ *v* the corresponding value in control rats), but was observed throughout the entire time course of the infection ($P < .0005$ *v* control values). The anti-SCF treated rats also exhibited significantly reduced levels of RMCP II in the jejunal mucosa at all time points tested (Fig 3A), as well as over the entire time course of the infection ($P < .0005$ *v* control values). This reduced intestinal concentration of RMCP II was paralleled by a highly significant depression of RMCP II levels in the blood at 6 or 10 days after infection ($P < .001$ at either interval, $P < .0005$ over the entire course of the infection; Fig 4A). At 14 days after infection, the anti-SCF–treated rats developed MMC hyperplasia and increases in jejunal and serum levels of RMCP II, perhaps partly as a result of the development of an antibody response that diminished the efficacy of the

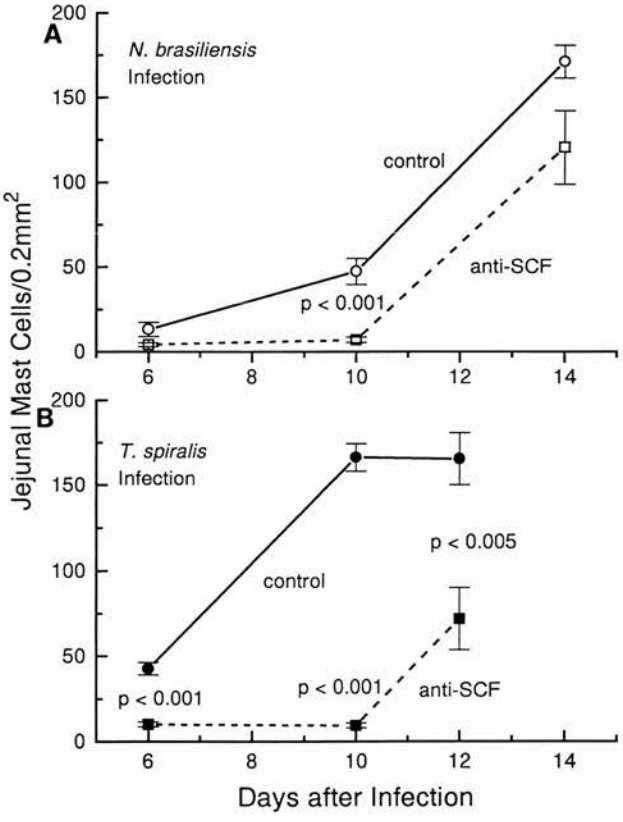

Fig 2. Mast cell densities in jejunum of rats infected with (A) *N brasiliensis* and treated with anti-rrSCF[164] (□) or normal sheep IgG (○), or (B) *T spiralis* and treated with anti-rrSCF[164] (■) or normal sheep IgG (●) (n = 5 for each interval in each group).

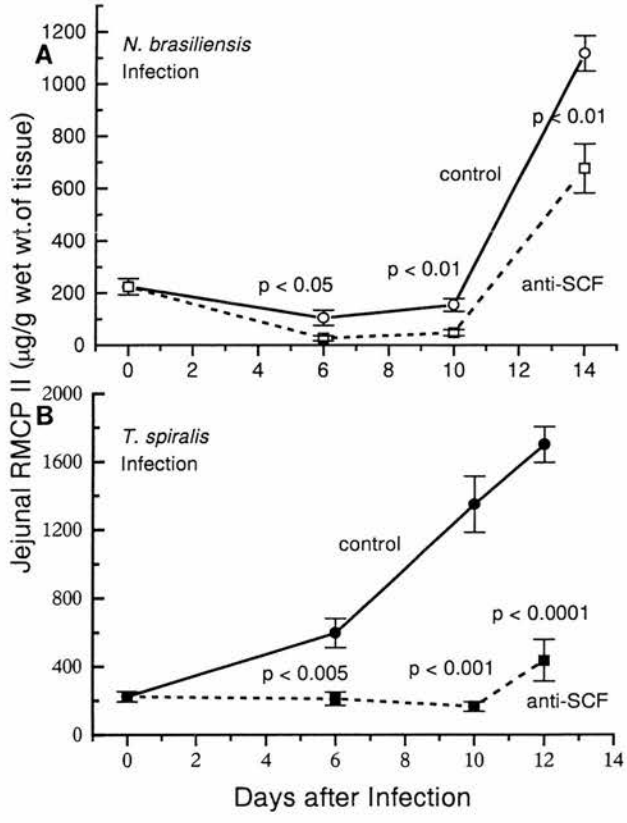

Fig 3. RMCP II content of jejunum of rats infected with (A) *N brasiliensis* and treated with anti-rrSCF[164] (□) or normal sheep IgG (○), or (B) *T spiralis* and treated with anti-rrSCF[164] (■) or normal sheep IgG (●) (n = 5 for each interval in each group).

sheep anti-SCF antibodies. Simple regression analysis of mast cell densities and RMCP II concentrations in the jejunal mucosa of all *N brasiliensis*–infected animals gave a highly significant correlation ($R = .96$, $P < .0001$, Fig 5A).

To determine whether SCF remains a significant factor in MMC hyperplasia or survival even after the onset of the intestinal MMC response, groups of rats were treated with anti-SCF or normal sheep IgG daily from day 10 of infection, when mast cell hyperplasia was well under way (eg, see Fig 2A). The rats treated with anti-SCF showed a 32% depletion of RMCP II from the small intestine on day 14, compared with values for normal sheep IgG-treated controls ($P < .01$; Table 4), with a concomitant 32% decrease in mast cell density ($P < .03$; Table 4). The 15% decrease in serum RMCP II concentrations in anti-SCF-treated rats was not significant (Table 4), nor were there any significant alterations in hematological parameters as a result of the anti-SCF treatment (Table 5).

Infection with T spiralis. Infective *T spiralis* larvae establish themselves in the intestine within hours of oral challenge, therefore treatment with anti-SCF was first given at the time of infection on day 0. Mast cell hyperplasia occurs sooner during trichinosis than in *N brasiliensis*–infected rats, and anti-SCF treatment significantly depressed mast cell densities on day 6, 10, and 12 of infection ($P < .001$, $P < .001$, and $P < .005$, respectively; $P < .0005$ over the entire course of the infection; Fig 2B) when compared with values in normal IgG-treated controls. Again, this result was paralleled by values for mucosal concentrations of RMCP II, which were significantly reduced 6, 10, or 12 days after infection ($P < .005$, $P < .001$, and $P < .001$, respectively; $P < .0005$ over the entire course of the infection; Fig 3B) in anti-SCF–treated rats. The relationship between mucosal RMCP II and mast cell densities, when analyzed by simple regression analysis, was significantly correlated ($R = .90$, $P < .0001$, Fig 5B). In contrast with responses in *Nippostrongylus*-infected rats, the systemic secretion of RMCP II was depressed on day 6 ($P < .001$), but not at other time points (Fig 4B). As previously reported,[10] we found that *T spiralis* infection, in comparison to *N brasiliensis* infection, was associated with a greater, and earlier, systemic release of RMCP II (compare Fig 4A and B).

Effects of treatment with rrSCF^{164} or anti-SCF on parasite fecundity. The effects of the administration of rrSCF^{164} or anti-SCF on the fecundity of *N brasiliensis* was determined by monitoring egg output in the feces of the rats. Egg output was maximal on day 6 of infection in both rrSCF^{164}-treated

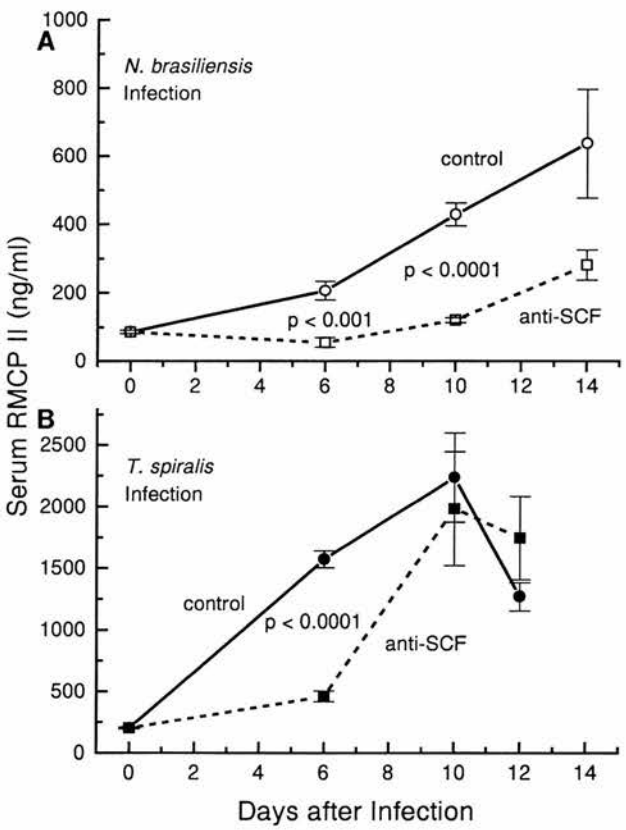

Fig 4. Serum concentrations of RMCP II in rats infected with (A) *N brasiliensis* and treated with anti-rrSCF^{164} (□) or normal sheep IgG (○) or (B) *T spiralis* and treated with anti-rrSCF^{164} (■) or normal sheep IgG (●) (n = 5 for each interval in each group).

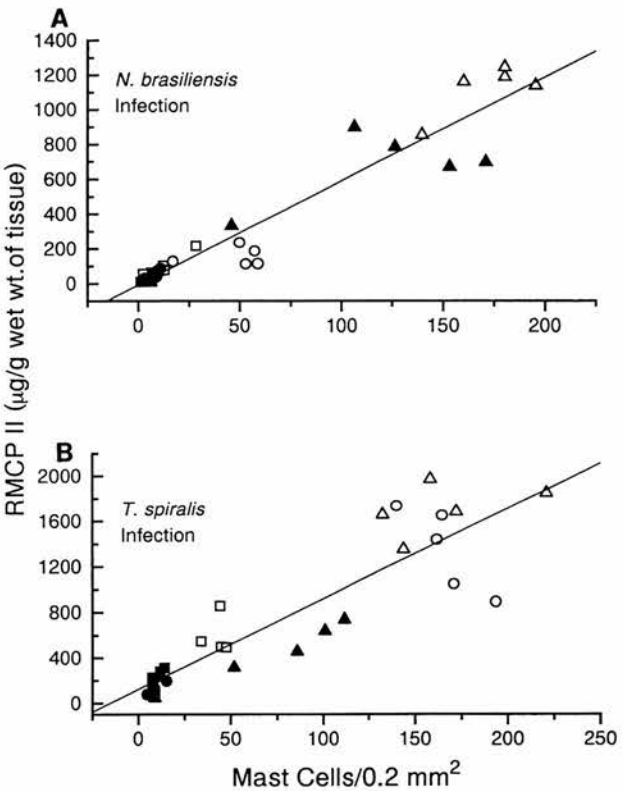

Fig 5. Correlation between mast cell densities and RMCP II concentrations in small intestine of rats infected with (A) *N brasiliensis* or (B) *T spiralis*. The data from control rats are represented by open symbols and those from anti-SCF treated rats by closed symbols (n = 5 for each interval in each group). (A) (■), D 6 anti-SCF; (□), D 6 control; (●), D 10 anti-SCF; (○) D 10 control; (▲), D 14 anti-SCF; (△), D 14 control. (B) (■), D 6 anti-SCF; (□), D 6 control; (●), D 10 anti-SCF; (○), D 10 control; (▲), D 12 anti-SCF; (△), D 12 control.

Table 4. Jejunal Mast Cell Densities and RMCP II Concentrations in Intestine and Serum in Rats Treated With Anti-SCF or Normal Sheep IgG (Control) From Day 10 to 13 of *N brasiliensis* Infection

	Day 0	Day 10	Day 14 Control	Day 14 Anti-SCF
Jejunal mast cells (cells/0.2 mm^2)	ND	ND	65 ± 7	44 ± 3*
Jejunal RMCP II (μg/g wet wt)	ND	ND	1,752 ± 106	1,186 ± 111†
Serum RMCP II (ng/mL)	95 ± 4	360 ± 21	452 ± 62	386 ± 121

Each of the groups contained five rats that were killed 24 hours after the last injection of anti-SCF or normal sheep IgG. Thus, n = 10 for day 0 and day 10 values (after blood was drawn on day 10, 5 of the 10 rats received anti-SCF treatment, and the other 5 received normal sheep IgG treatment), whereas n = 5 for the day 14 values.

* $P < .03$.

† $P < .01$ by two-tailed Student's *t*-test *v* day 14 control values.

and vehicle-treated (control) rats and diminished until no eggs were detected by day 11 (Fig 6A). While values for fecal egg output were greater in the rrSCF^{164}-treated as opposed to the control rats at many intervals of the infection, these differences did not achieve statistical significance.

In the experiments in which rats were treated with anti-SCF or normal sheep IgG, the worm egg output was monitored over the latter part of the infection to determine whether depressing the intestinal mucosal mast cell response would also lead to an alteration of the normal worm expulsion kinetics. Interestingly, we found that the animals that were treated with anti-SCF (and had reduced densities of jejunal MMCs) had significantly lower fecal egg-counts on both days 8 and 9 than did the normal IgG-treated controls ($P < .03$ at both intervals, Fig 6B). Over the entire time course of the response shown in Fig 6B, values for anti-SCF treated rats were significantly lower than those in control rats by ANOVA ($P = .001$). However, egg output had virtually ceased by day 11 in both groups.

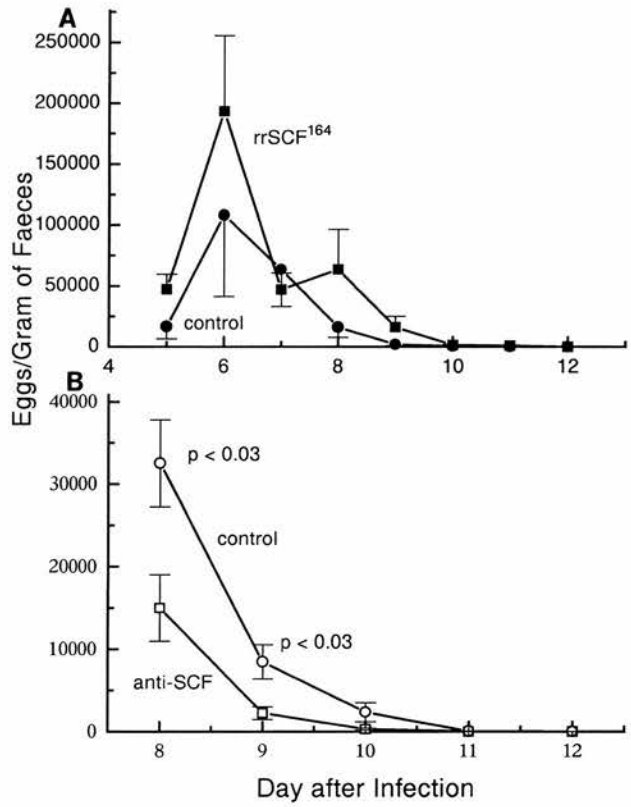

Fig 6. Fecal egg-output from *N brasiliensis*–infected rats treated with (A) rrSCF^{164} (■) or vehicle (control) (●) or (B) anti-SCF (□) or normal sheep IgG (control) (○) (n = 5 for each interval in each group except for days 10, 11, and 12 in (A), in which n = 4 for these three intervals in both groups).

DISCUSSION

Our findings provide additional evidence that SCF importantly contributes to the MMC hyperplasia that occurs during nematode infection in rats, show that this response can be markedly suppressed by administration of an anti-SCF antibody, and raise interesting questions about the role of SCF and mast cells in the regulation of parasite fecundity during *N brasiliensis* infection.

In confirmation of our previous study,[14] we found that treatment of uninfected rats with rrSCF^{164} at 25 μg/kg/d, intravenous (IV) for 14 days produced a significant (85%) increase in jejunal MMC numbers. However, in contrast to our previous study,[14] the elevation in jejunal RMCP II content in rrSCF^{164}-treated versus vehicle-treated rats did not achieve statistical significance. On the other hand, rrSCF^{164}

Table 5. Hematologic Parameters in Rats Treated With Anti-SCF or Normal Sheep IgG (Control) From Day 10 to 13 of *N brasiliensis* Infection

	No.	WBC	RBC	Hb (g/dL)	Hct %
		($\times 10^{10}$/L)	($\times 10^{12}$/L)		
Day 0	10	1.4 ± 0.05	6.7 ± 0.1	15.7 ± 0.2	40.0 ± 0.7
Day 10	10	1.1 ± 0.11	7.1 ± 0.2	16.1 ± 0.3	39.2 ± 1.2
Day 14 control	5	1.2 ± 0.05	6.7 ± 0.1	15.1 ± 0.3	40.4 ± 0.8
Day 14 anti-SCF	5	1.1 ± 0.12	6.4 ± 0.1	14.5 ± 1.6	37.4 ± 4.2

Rats were killed 24 hours after the last injection of anti-SCF or normal sheep IgG. Day 0 and day 10 values are from all 10 rats (the 5 that later received anti-SCF treatment and the 5 that later received normal sheep IgG treatment; the treatments began after blood was drawn on day 10).

treatment had only modest effects on the changes in jejunal MMC numbers, or on the jejunal or serum levels of RMCP II, that were associated with *N brasiliensis* infection. Compared with vehicle-treated rats, rrSCF^{164}-treated rats had higher levels of jejunal MMCs and significantly higher levels of jejunal RMCP II at day 8 of infection. Also, the peak mean levels of serum RMCP II occurred earlier (day 8 *v.* day 10) and reached a roughly twofold higher maximum in rrSCF^{164}-versus vehicle-treated rats. These findings indicate that administration of exogenous SCF can increase the kinetics and magnitude of jejunal MMC development and RMCP II production during *N brasiliensis* infection. However, later during the infection, numbers of jejunal MMCs and serum levels of RMCP II were quite similar in rrSCF^{164}- and vehicle-treated rats, and, due to the broad range of individual values, the higher mean levels of RMCP II in the jejunal mucosa of rrSCF^{164}-treated rats were not significantly different than those in the vehicle-treated controls. One possible explanation for these findings, which we favor, is that endogenous levels of SCF in normal rats are sufficient to permit near maximal MMC hyperplasia (presumably, in response to T-cell–associated cytokines) in the setting of *N brasiliensis* infection.

To assess the importance of endogenous SCF in the intestinal MMC response to nematode infection in normal rats, we administered a polyclonal sheep antirat SCF antibody to rats infected with *N brasiliensis* or *T spiralis,* as well as to normal rats. Treatment of normal rats with anti-SCF for 4 days totally ablated the intestinal MMC population and virtually eliminated circulating RMCP II; it also significantly reduced numbers of peritoneal mast cells and diminished numbers of all circulating white blood cells, probably because of an effect on hematopoietic progenitor cells. Treatment with anti-SCF beginning at the time of infection profoundly interfered with mast cell hyperplasia in the jejunum and significantly diminished the systemic release of RMCP II after infection with either *N brasiliensis* or *T spiralis.* These effects were most marked at the earlier intervals of infection, probably because of the later development of antibodies to sheep IgGs in the anti-SCF–treated rats. When anti-SCF was administered to rats that already had undergone expansion of jejunal MMCs in response to *N brasiliensis* infection, anti-SCF treatment resulted in a significant decrease in both intestinal RMCP II concentrations and mast cell numbers, but produced neither a significant depression of RMCP II concentrations in the serum nor a significant change in numbers of peripheral blood white cells.

One interpretation of our findings with anti-SCF is that endogenous SCF is necessary for the survival of mast cell populations, including both baseline populations of MMCs in uninfected rats and the markedly expanded MMC populations in nematode-infected rats. rrSCF^{164} can maintain mouse mast cell survival in vitro[18,19] or in vivo[19] by suppressing apoptosis, and the abrogation of this effect by anti-SCF could account for our findings. Of course, rrSCF^{164} can promote the recruitment of mast cell precursors in vivo[14,20] and can favor their local development into mature mast cells. It also can synergize with IL-3 to promote rat mast cell proliferation.[16,17] These additional effects of SCF also may have been antagonized by anti-SCF treatment, particularly when treatment was begun at the time of infection.

However, the total ablation of mature MMCs from the intestinal mucosa of normal rats by anti-SCF cannot easily be explained by a suppression of recruitment of mast cell precursors. The half-life of mucosal mast cells in rat intestine has been estimated at around 40 days,[21] and blocking recruitment for only 4 days would be expected to have a negligible effect on mast cell numbers. Nor can an effect solely on recruitment of precursors readily explain the depletion of MMCs from the intestinal mucosa that was observed when anti-SCF treatment was started on day 10 of infection, when T-cell–driven mastocytosis was already well established. These findings can, perhaps, best be explained by effects of anti-SCF treatment on the SCF-dependent suppression of mast cell apoptosis.

In *T spiralis* infection in mice, treatment with an anti–c-*kit* receptor antibody not only diminished intestinal mast cell hyperplasia, but also reduced spontaneous parasite expulsion.[13] This finding supports the widely held view that mast cells can represent an important component of host immunity to parasite infection. Yet we found that treatment with rrSCF^{164} which accelerated the development of the MMC response, did not diminish and actually somewhat increased parasite egg production during *N brasiliensis* infection. This effect was particularly evident at day 8 of infection, the day on which rrSCF^{164}-treated rats had significantly more jejunal MMCs than did control rats (Table 1). Moreover, treatment with anti-SCF, which markedly suppressed the MMC hyperplasia associated with *N brasiliensis* infection, substantially (by >50%) and significantly ($P = .001$) diminished *N brasiliensis* egg production during the later stages of the infection.

In considering these findings, it should be noted that genetically mast cell-deficient (*W/W^v, Sl/Sl^d*) mice or (*Ws/Ws*) rats exhibit little or no impairment in their ability to expel a primary infection with *N brasiliensis* (reviewed in[11,12]), findings which suggest that mast cells are not an essential component of the host immune response to this nematode. Indeed, Arizono et al[12] found that, at the peak of egg output on day 8 of *N brasiliensis* infection, parasite egg production was ~3.5-fold greater in the congenic +/+ normal rats than in mast cell-deficient *Ws/Ws* rats (133,300 *v.* 38,100 eggs/g of feces, $P < .01$); egg production in both the *Ws/Ws* and +/+ rats declined rapidly thereafter, so that there was no significant difference in the low levels of egg output observed on day 11 of infection.

Both the findings in *Ws/Ws* and congenic +/+ rats[12] and our findings in normal rats raise the interesting possibility that mast cells (and/or other SCF-responsive cells) may actually have some effects that favor parasite fecundity during *N brasiliensis* infection. For example, perhaps the activation of increased numbers of intestinal MMCs for mediator release, and the resulting increased permeability of gut blood vessels and intestinal mucosa, promotes the nutrition of the parasites. Whatever mechanism(s) might account for the apparent increased fecundity of *N brasiliensis* in rats treated with anti-SCF, it has been shown recently that suppression of IgE levels can decrease both the worm burden and the

parasite egg production associated with primary infection with *Schistosoma mansoni* in mice.[22] Taken together with our results and those of Arizono et al[12] in rats infected with *N brasiliensis,* these findings suggest that, in certain parasite infections, aspects of the mast cell- and/or IgE-dependent immune responses that are elicited by the organisms may have effects which are more advantageous to the parasite than to the host.

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